Title: DETERMINING THE EFFECT OF TEMPERATURE ON SPECIES INTERACTIONS IN MICROCOSMS: A QPCR APPROACH

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DETERMINING THE EFFECT OF TEMPERATURE ON SPECIES INTERACTIONS IN MICROCOSMS: A QPCR APPROACH

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Contents

Abstract

Predicting the impact of environmental change is a major goal and challenge in ecology. With climate change threatening the biodiversity and ecosystem functioning of our natural ecosystems, understanding the effects of such change and how these are mediated through a community is of critical importance. Community stability could be severely affected by temperature through extinctions, alterations in species dominance and species-specific responses.

One approach to testing the consequences of climate change on a community is to manipulate experimental aquatic microcosms. However, investigating community-level responses to change in experimental microcosms has been limited by the ability to accurately monitor the basal trophic level of bacteria. Here I develop a molecular approach to monitoring bacteria by using qPCR. The qPCR approach was successful for three bacterial species and produced sensitivity to the single cell.

The qPCR approach was implemented in an experimental setting to aid the investigation of the relationships between temperature, species interactions and community properties. Direct temperature response was species-specific, but indirect interactions strongly mediated temperature through the community, altering competitor and predator response. Therefore, predicting species and community response to environmental change is dependent on knowledge of specific-species response, indirect pathways of interaction and the effects of community composition.

Acknowledgements

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1 Introduction

1.1 Overview

This chapter reviews the existing literature investigating ecological communities and the processes through which they operate. As such definitions of ecological properties are explored and the methods used to investigate the mechanistic hypotheses are analysed. Ecological communities have been interpreted as food chains and webs, mathematically modelled and examined in field and laboratory investigations. Laboratory microcosms provide the ideal testing ground for ecological theory by using model organisms. Although generally the basal trophic level of bacteria is less well monitored due to practical methodological constraints. Using the food web approach has given rise to a set of terminology and has advantages and disadvantages for interpretation. The food web approach inherently places focus upon direct trophic interactions, however the importance of indirect interactions is beginning to be recognised. Understanding the processes within ecological communities is important for its own intrinsic value of interest, but is also hugely important for ecological services, biodiversity and conservation. The threat of climatic and environmental change emphasises the critical need to understand our ecological communities and the processes that control community response.

1.2 The use of experimental laboratory microcosms

Microcosm studies of ecological processes are constructed ecosystems which allow the controlled experimentation of ecological systems under physical and biological constrains to develop a greater understanding of ecological principles and processes. The use of microcosms as an experimental tool has many advantages. The laboratory construction allows a very high level of control and manipulation of environmental and biological factors. Also due to the small-scale nature of microcosms the experiments have a very high repeatability. Drake and Kramer (2012) reason that the ecological scale of ecological microcosms is sufficiently accurate due to the short generation time of the organisms used. Cadotte *et al.,* (2005) recognises that whilst thousands of generations can easily be observed in the laboratory, to accomplish the same feat in the field would take several generations of ecologists.

The chief criticism of ecological microcosms is their lack of realism and generality (Carpenter, 1999). It is argued that microcosm investigations cannot be extrapolated into the natural world and are therefore largely irrelevant to studying natural ecosystems (Carpenter, 1999). However, laboratory microcosms are largely used to investigate aspects of theory, with the aim of developing and extrapolating theoretical concepts. Microcosms have also been criticised as ecologically simplistic. It is commonly viewed by critics that properties of natural systems are in part a result of their complexity, therefore to reduce this complexity, the capacity for reliable predictions is also reduced. However, the advantages of microcosm experiments can be attributed to their simplicity and permit the investigation of intact communities, which cannot be accomplished in the field (Cadotte *et al.,* 2005). Their simplicity allows the development of a mechanistic understanding. Many microcosm investigations aim to identify ecological processes and understand how they can work. Therefore, microcosms reveal the potential mechanisms and responses and enable the identification and testing of causal theories (Drake and Kramer, 2012). If theory fails to withstand testing under controlled circumstances, then the likelihood theory would hold in nature must be questioned. Moreover, it is much faster and less costly to realise the flaws and failures of theory in microcosms investigations opposed to field experiments (Drake and Kramer, 2012).

Experimental microcosms have been constructed using bacteria and protists and used to investigate a range of ecological principles, shown in table 1.1. These organisms are used as a model species (see Montagnes *et al.,* 2012). The primary focus of these studies is generally placed upon the dynamics of the protists, leaving the bacteria dynamics overlooked. The bacterial dynamics are often regarded as not relevant due to the faster timescale at which they operate. Within the food web, bacterial species are often aggregated into a bacterial black box. This aggregation may hide important dynamics and species interactions. Ings *et al.,* (2009) discusses the advantages of an individual-based network resolution, and how this approach may enable a mechanistic basis incorporating the MTE and foraging theory (Petchey *et al.,* 2008). Considering the aim of a comprehensive understanding of these systems, overlooking the bacterial trophic level may result in misleading conclusions. However, the ability to regulate and monitor this trophic level has been limited by the time-consuming approach of current methods. Chapter 2 therefore explores the development of a molecular approach to resolve this issue. The molecular approach developed is implemented in Chapter 3. Petchey *et al.,* (2010) states that there are few models that consider how environmental variables, particularly temperature, impact community structure and concludes that our understanding of the effects of temperature upon community structure urgently needs substantial development. Chapter 3 therefore aims to apply the molecular method in an ecological context to understand the relationships between temperature, species interactions and community structure.

Table 1.1: Experimental laboratory microcosm studies, the organisms utilised (bacteria and protists) and the manipulated variable, continued on page 10.

Study	Bacteria	Protists	Manipulated Variables
Balciunas and Lawler (1995)	Unknown	*Chilomonas paramecium, Colpidium striatum, Euplotes patella*	Resource availability and number of trophic levels
Holyoak and Lawler (1996a, 1996b)	Unknown	*C. striatum, Didinium nasutum*	Dispersal
Fox and Olsen (2000)	*Serratia marcescens, Bacillus cereus, Bacillus subtilis*	*Paramecium tetraurelia, C. striatum, D. nasutum*, (and flagellate *Chlamydomonas reinhardtii*)	Complexity (linear or reticulated food web structure)
Fox and Morin (2001)	*S. marcescens, B. cereus, B. subtilis*	*C. striatum*, *Tetrahymena thermophila, Blepharisma americanum, P. tetraurelia, D. nasutum E. patella*	Density dependence of intra/interspecific interactions and temperature
Jiang and Morin (2004)	*S. marcescens, B. cereus, B. subtilis*	*C. striatum, P. tetraurelia*	Temperature and competition
Jiang and Kulczyki (2004)	*S. marcescens, B. cereus, B. subtilis*	*C. striatum, P. tetraurelia, D. nasutum*	Temperature and species interaction (competition or predation)
Scholes *et al.,* (2005)	Unknown	*Paramecium caudatum, C. striatum, Colpidium colpidium, Tetrahymena. pyriformis, Colpoda cucculus, Halteria sp. Loxocephalus sp. C. paramecium.*	Energy availability and disturbance (temperature shock)

Study	Bacteria	Protists	Manipulated Variables
Fox and Barreto (2006)	*B. subtilis, Escherichia coli, Micrococcus roseus, Micrococcus luteus, Pseudomonas fluorescens, Enterobacter aerogenes, Aloaligenes eutrophus, Staphylococcus epidermis, chromobacterium lividum, Bacillus megaterium, Aquaspirrillum serpens, S. marcescens*	*C. striatum, T. thermophila*	Coexistence by adjusting number of bacterial species
Worsfold *et al*., (2009)	*S. marcescens, B. cereus, B. subtilis*	*P. caudatum, Paramecium bursaria, T. pyriformis, C. cucculus, D nasutum, Stentor, E. patella, Blepharisma japonicum*	Predator removal
Cooper *et al*., (2012)	*S. marcescens*	*P. caudatum, D. nasutum*	Landscape fragmentation
Clements *et al*., (2013)	*S. marcescens, B. cereus*	*B. japonicum, P. caudatum, Loxocephalus sp.*	Assembly order and temperature
Griffiths *et al.,* (2015)	*S. marcescens*	*B. japonicum, P. caudatum*	Salinity, Productivity, Temperature.
Hammill *et al.,* (2015)	Unknown	*Paramecium aurelia, E. patella, Philodina roseola* (and flatworm: *Stenostonum virginianum*)	Indirect interactions through the presence/absence of nonprey species

1.3 Environmental change

Over the past 100 years the Earth's climate has warmed approximately 0.6°C (Walther *et al.,* 2002) and current predictions suggest that over the next 100 years global temperatures will rise between 0.3°C and 4.8°C (IPCC, 2013). There is evidence for ecological impacts, (eg. phenology, range shifts) due to recent climate change (Walther *et al.,* 2002, Winder and Schindler, 2004, Killengreen *et al.,* 2007). However there remains an element of uncertainty in future climate change estimates. Nonetheless climatic change is likely to have a profound effect upon ecological communities through the impact upon individual species and the impact on the links within ecological networks (Walther, 2010, Woodward *et al.,* 2010a). The principal components of ecological networks, such as connectance, species richness and productivity (Warren, 1990, Vermaat *et al.,* 2009) may also be influenced by climatic and environmental change (Petchey *et al.,* 2010). These network properties and thus the robustness and stability of these networks are likely to be strongly affected by climate change (Woodward *et al.,* 2010a). However, how communities, and the biodiversity and the ecosystem services associated with them will change under climatic and environmental change is poorly known (Pimm, 2009, Montoya and Raffaelli, 2010). It is thus immensely important to develop an understanding of how food-webs respond to climatic and environmental change and the underlying mechanisms that mediate responses within ecological networks. This need to understand and predict how ecological responses are mediated through the system has led ecologists to explore the effects of individual environmental parameters upon food-webs.

It should be noted that environmental factors rarely act individually in nature, yet the responses to these factors are primarily considered in isolation and the interactions between environmental factors are often not considered (Griffiths *et al.,* 2015). The combined effects of multiple environmental factors may be non-additive (Walther, 2010, Griffiths *et al.,* 2015) and therefore community level response could be wrongly predicted if based upon studying factors independently. However, to deconstruct the interactions between environmental conditions and communities, a mechanistic understanding of the community response to each factor in isolation is required. Thus

investigations that contemplate environmental conditions independently aid the disentangling of processes.

1.3.1 Metabolic Theory of Ecology (MTE)

The Metabolic Theory of Ecology (MTE) defines the metabolic rate of organisms as the fundamental biological rate which governs ecological processes. This theory has therefore been put forward as the foundation for building a mechanistic understanding of ecological processes. The MTE describes the effects of body size and temperature upon the metabolic rate of organisms and how these effects are mediated through flows of energy and matter in populations, communities and ecosystems (Brown *et al.,* 2004). Metabolic rate has been recognised as an important biological rate for community processes as it could govern the rates of growth, consumption and the life history schedule and thus impact species interactions and network dynamics (Gillooly *et al.,* 2002, Brown *et al.,* 2004). Body size and temperature have been identified as the two most important variables that control biological rates (Gillooly *et al.,* 2001, Clarke, 2006). Brown *et al.,* (2004) state that metabolic theory predicts how metabolic rate, which is body-size- and temperature- dependent, determines the rates of resource uptake and resource allocation, thus controlling the rates of consumption and population growth and ultimately governing ecological processes from the individual to the ecosystem. Therefore the MTE could provide a mechanistic basis for understanding the effects of temperature upon communities.

The MTE has been used as a foundational basis for mathematical models alongside the Arrhenius equation to determine the temperature dependence of functional response (Englund *et al.,* 2011) and could be extended to model other biological rates that are coupled with metabolic rate, such as developmental rate (Gillooly *et al.,* 2002). Although the use of the MTE as a framework for modelling biological rates has found support and contradiction within models and empirical research. Empirical evidence is highly desirable to support the parameters produced by the theoretical use of the MTE.

1.4 Indirect interactions

The inherent focus on trophic interactions in investigations of food web dynamics and structure has to an extent eclipsed the potential significance of non-trophic interactions and indirect effects (Berlow *et al.,* 2004). Indirect effects transpire when one species impacts a second species without necessarily directly interacting, generally via resources or another species. This can occur through a series of direct interactions, changes to environmental conditions and resource availability or when the interaction between two species is adjusted by a third species (Wootton, 1994). It is essential to keep in mind that indirect effects can operate through multiple pathways simultaneously (Spiesman and Inouye, 2015). By only considering direct pathways or just a singular indirect pathway, conclusions may be overly simplistic. Yet indirect pathways, such as predator-mediated and resource-mediated indirect effects, are often considered independently. However, Spiesman and Inouye (2015) suggest that when these pathways occur together the net effect is not the same as summing the effect of both pathways, therefore indirect pathways can interact and adjust the strength of indirect interactions.

There is no conclusive evidence that direct or indirect effects are stronger than the other (Fox and Olsen, 2000). Wootton (1994) expects that direct effects are stronger because some indirect effects could rely on the occurrence of strong direct effects and may be weakened by a high level of environmental variation. On the other hand, indirect interactions could drive the response of communities to perturbations, and the pattern of indirect pathways could determine community stability. Thus Rall *et al*., (2010) suggests indirect effects could be more important than direct effects. In the face of climatic change, there is a greater urgency to understand the impact of indirect interactions. Moreover indirect interactions may offset or exacerbate the effects of direct interactions, in particular simulation studies suggest that indirect effects could help to stabilise communities (Wootton, 1994). Furthermore if indirect effects are common in natural ecosystems, then species invasions or removals could have a widespread effect (Wootton, 1994, Worsfold *et al.,* 2009). Thus the development of theory and a deeper understanding and of indirect interactions could have a major impact on the development of policy and management strategies, particularly within conservation programmes. Shurin *et al.,* (2012) emphasise the need to understand how

indirect effects are mediated through the food web and how they relate to direct effects to develop comprehensive and robust models of ecosystems. Therefore, resolving the interface of direct and indirect interactions is integral to developing a mechanistic understanding of food-webs, and community response to perturbations, including environmental change, invasive species and extinctions.

Winder and Schindler (2004) argue that understanding the effects of climate change upon species interactions is the largest challenge in forecasting projections, as species show unique responses to environmental change. Chase *et al.,* (2002) provides a comprehensive review of theoretical and empirical research studying the interaction between predation and competition. However, the literature appears contradictory as predation is suggested to increase, decrease or have no effect upon the strength or impact of interspecific competition. By testing the effects of the loss of predators in an exposed rocky shore community, O'Connor *et al.,* (2013) found that the loss of predators had indirect negative effects on species diversity and the cascading effects were mediated by intermediate consumers. Additionally, Worsfold *et al.,* (2009) found that the effects of loss of predator are highly context-dependent. These studies highlight the importance of considering the coupled effects of trophic cascades and non-trophic interactions.

1.4.1 Food webs and community properties

Food webs describe predator-prey interactions of organisms in a community. Historically food webs have been used as summaries of patterns of trophic interactions within a community as well as descriptive devices (Morin, 2011). The use of food webs has advanced the depiction and understanding of trophic networks, developed the creation of models and repeatable patterns have emerged (Morin, 2011, Thompson *et al.,* 2012). Food webs can also provide a framework for disentangling the complex relationships between patterns and ecological processes (Thompson *et al.,* 2012). However, when considering food web theory, it is important to recognise the potential drawbacks, adjusted from Morin (2011) and listed below.

1. The interactions considered in food webs are primarily trophic based. This approach could lead to the nature and importance of non-trophic interactions also being overlooked.
2. Food webs inherently focus upon interspecific interactions, therefore intraspecific interactions and mutualisms are also typically overlooked.
3. In nature it can be challenging to account for all species in a community and to be certain all species are correctly defined taxonomically and interactions accurately represented.
4. If nodes are used to represent aggregates of species, caution must be taken to ensure results and conclusions are not artefacts of this approach as oppose to biological reality (Dunne *et al.,* 2002).

With these limitations in mind, studies have recognised the potential importance of indirect interactions, such as apparent competition and trophic cascades, and food web properties which link to complexity and stability. These are discussed below. Furthermore, the aggregation of species into one node within a food web, happens often in laboratory microcosms where bacteria species are grouped together and treated as a non-interactive resource. Unpacking this aggregation and understanding species dynamics at the basal trophic level would be highly advantageous.

1.5 Developing microcosms as a tool

Laboratory microcosms manipulating experimental aquatic communities have provided a framework for testing ecological theory. In the context of exploring food web properties, and developing a comprehensive mechanistic understanding of the community, all individual components of the community must be monitored and understood. Many of these questions require the development of an experimental tool to aid the comprehensive monitoring of all species, to fully resolve species dynamics and interactions. In Chapter 2, I develop a molecular approach using a qPCR technique to enumerate the bacteria. This technique enables the monitoring of the bacterial trophic level within experimental microcosms, which would benefit our comprehensive understanding of these experimental communities. In Chapter 3 I implement the use of qPCR within an experimental setting, to investigate the effects of temperature and community composition and the potential interaction of these factors.

2 Molecular Methods

2.1 Overview

This chapter details the development of a molecular methodology to detect and enumerate bacteria in ecological microcosm experiments. Microcosms offer the opportunity to test ecological theory. Experimental communities are constructed with protozoa forming the higher trophic levels and it is usually these species dynamics that are monitored. Bacteria such as *Pseudomonas fluorescens, Bacillus cereus,* and *Serratia marcescens* are often used within ecological microcosms as a food source for higher trophic levels (Fox and Barreto, 2006, Clements *et al.,* 2013, Altermatt *et al.,* 2015). Yet this bottom bacterial trophic level is generally not monitored or regulated. However, it is suggested that bacterial dynamics have an impact on population dynamics, species interactions and community processes (Jiang and Morin, 2004, Krumins *et al.,* 2006, Fox and Barreto, 2006). Therefore, better defined and more accurate quantification of bacteria is essential to facilitate the understanding of the effects of bacteria upon the food web.

Quantifying bacteria has traditionally depended upon counting the number of colony forming units in serial dilutions on agar plates (see Jiang and Morin, 2004). The problems with this method to detect and enumerate bacteria are firstly it is time-consuming and

labour intensive and secondly the species specific identification of bacteria species can be unclear (Franklin *et al.,* 2001). This second issue could be overcome with a known, well-defined starting point for community assembly. However, this is still vulnerable to contamination, so exact species specific classification of the bacterial assemblage can still be uncertain (Cochran-Stafira and von Ende, 1998, Jiang and Morin, 2004).

Molecular methods have been used to detect and quantify bacteria in soil ecology (Zhou *et al.,* 1996, Hermansson and Lindgren, 2001, Stubner, 2002, Kolb *et al.,* 2003), water treatment (Toze, 1999, Zhang *et al.,* 2009, Kim *et al.,* 2013) and food microbiology (Furet *et al.,* 2004, Petri *et al.,* 2013, Bottari *et al.,* 2013). In soil ecology, qPCR has been used to target 16S ribosomal DNA (rDNA) of methanotrophic bacteria (Kolb *et al.,* 2003), ammonia oxidising bacteria (Hermansson and Lindgren, 2001) and of a specific organism lineage such as *Desulfotomaculum* (Stubner, 2002). Ammonia oxidiser specific 16S rDNA has also been used in water treatment work for PCR- denaturing gradient gel electrophoresis (DGGE) (Zhang *et al.,* 2009). DGGE is a molecular fingerprinting technique, which describes the community diversity, but cannot quantify the abundance of specific species. Reviews by Toze (1999) and Kim *et al.,* (2013) provide more detail on the development and application of molecular techniques utilised in water and wastewater research, including clone libraries, molecular fingerprinting, hybridization and the potential of qPCR. In food microbiology, studies have investigated the use of molecular methods to detect lactic acid bacteria in milk, wine and cheese (Furet *et al.,* 2004, Petri *et al.,* 2013, Bottari *et al.,* 2013). For example, Petri *et al.,* (2013) detected 13 specific lactic acid bacteria species in wine through nested PCR but cannot quantify their abundance. Whilst Bottari *et al.,* (2013) developed a qPCR for lactic acid bacteria in cheese which can determine the relative abundance of each species but not absolute abundance. Additionally, molecular techniques have enabled a more detailed description of bacterial assemblages in studies investigating biodiversity and biogeography by improving detection and classification (Muyzer, 1999, Horner-Devine *et al.,* 2003). These studies show that molecular methods to detect and quantify bacteria have a broad application in ecology. However, many studies either monitor bacteria dependent upon their function rather than species-specific detection, or produce a relative rather than absolute quantification of specific species. The point of this work is not just to detect, but also quantify absolute abundance of specific species known to be initially present in microcosms.

In a recent comprehensive review of sampling methods for microcosms (Altermatt *et al.,* 2015), serial dilutions and flow cytometry were described, however notably molecular methods were absent. This work tests and validates a new molecular methodology which will allow clear and precise detection and enumeration of a bacterial assemblage. Applying molecular methods in this experimental context would provide a greater resolution of the bacterial community, and aid understanding of species interactions and community response. Molecular methods would provide a less time-consuming and more replicable alternative to the current methods.

2.2 Molecular ecology and PCR

Polymerase chain reaction (PCR) is a technique commonly used in molecular biology to amplify a small segment of DNA to generate 'billions of copies' of the DNA sequence. The reaction works by combining the target DNA sample with the following reagents:

- DNA polymerase; a thermostable enzyme which synthesises the new DNA strands.
- Nucleotides, or dNTPs (deoxynucleotide triphosphates); the building blocks for the new strands of DNA.
- Primers; oligonucleotides which specify the target DNA sequence. Two primers are used to form a pair (the forward primer and reverse primer) which are complementary in the 5' to 3' direction to the target DNA sequence in each strand.
- Buffer solution; provides an optimum chemical environment for the DNA polymerase.

There are three clear steps within PCR; Denaturation, Annealing and Extension (fig.2.1). Denaturation occurs when the sample is heated to more than 90°C, thus separating the DNA strands by breaking the relatively weak hydrogen bonds between the nucleotides. At the second stage, the annealing stage, the sample is cooled to a temperature between 50°C - 60°C. The pair of primers then is able to bind (anneal) to each end of the target DNA sequence. In the third stage of the PCR, the extension phase, the sample tube is heated to 72°C (an ideal temperature for replication) and the nucleotides in the solution are added to the sequence specified between the primer pair by the polymerase to form a complementary strand of DNA. These three steps result in the duplication of the DNA sequence, creating two new strands of replicate DNA from one strand. The two new strands of DNA can then undergo the same process, producing four DNA strands. Therefore, the three stages form a cycle and each cycle doubles the amount of target DNA sequence, thus the reaction occurs exponentially. In 30 cycles more than one billion copies of the target DNA sequence can be generated. The PCR process is automated using a thermal cycler. To analyse the generation of the amplicon, the PCR products can be separated by size using agarose gel electrophoresis. The size of the fragments can then be determined using a molecular weight marker, referred to as a DNA ladder.

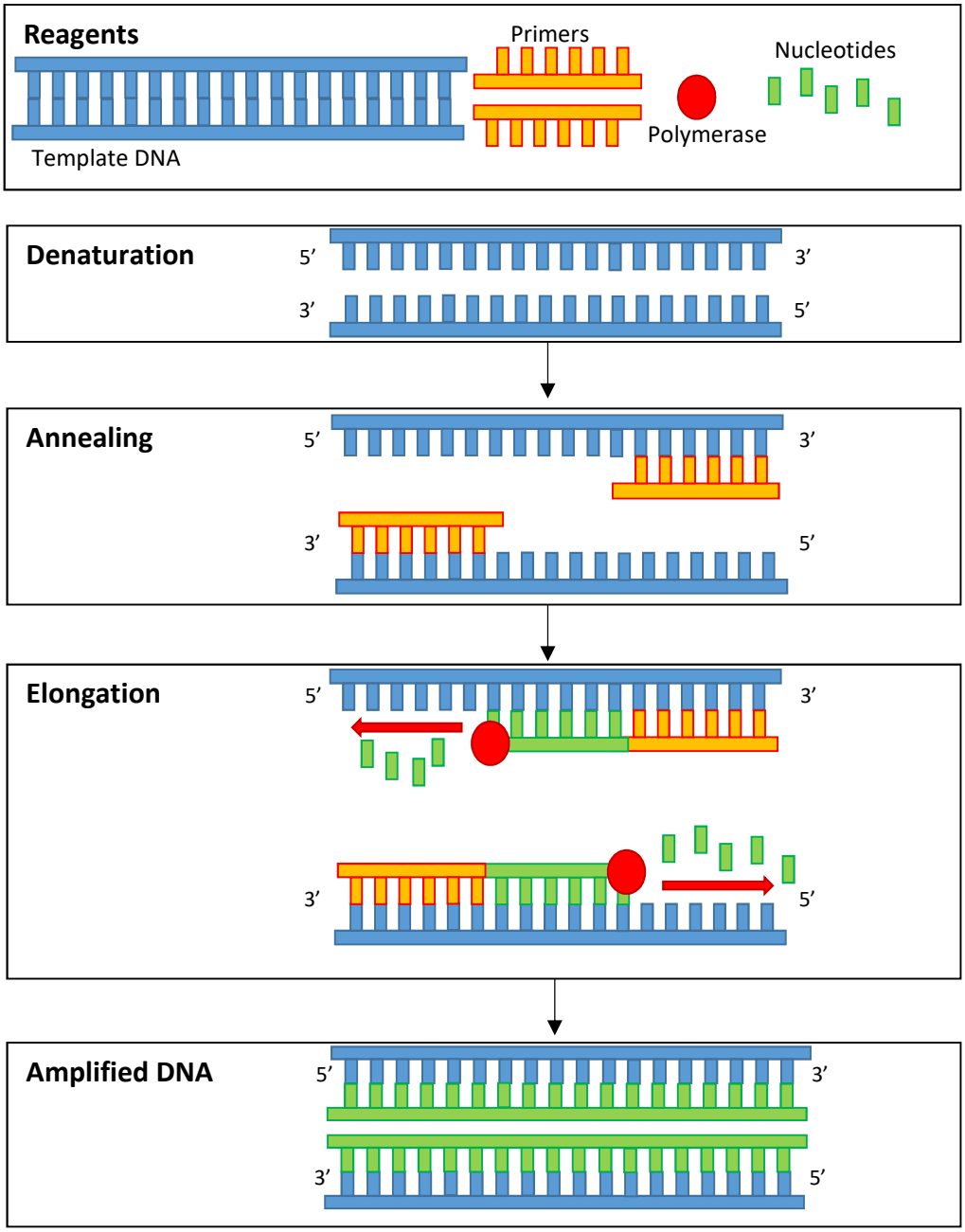


Figure 2.1: The reagents of PCR and the three stages; Denaturation, Annealing and Elongation. These three stages form one cycle. During denaturation the double stranded DNA separates (denatures). In the second stage, the primers anneal to the target DNA. During the elongation stage, the polymerase adds nucleotides to the primers to produce complementary DNA. At the end of each cycle, one strand of DNA is amplified in to two strands of DNA. This amplified DNA is called the amplicon.

qPCR (quantitative polymerase chain reaction) uses the same process as standard PCR, but with the addition of an intercalating fluorescent dye that binds specifically to double-stranded DNA. This allows the amplified DNA to be fluorescently labelled. Thus

during each cycle, the concentration of the amplicon can be directly quantified by the amount of fluorescence released relative to a standard in real time. As the amount of DNA increases with each cycle, the number of cycles needed to detect fluorescence is proportional to the amount of the original template DNA. This can be used to quantify the number of genomes present, and as one genome is present per cell, and bacteria are single-cell organisms, this provides a direct count for the bacteria. Intercalating dyes often used include SYBRgreen, Syto9, Evagreen and LC green. There have been reported issues with some of these dyes including dye instability, PCR inhibition, and low reproducibility during melting curve analysis (Monis *et al.,* 2005). For all qPCR reactions in this study the intercalating dye used was Syto9. Syto9 has been reported to have low inhibition and high reproducibility (Monis *et al.,* 2005, Gudnason *et al.,* 2007). Measuring the level of fluorescence at each cycle produces a sigmoidal curve with three phases (fig.2.2). The first phase shows an undetectable signal close to the baseline. The second phase shows exponential increase in fluorescence as the reaction doubles the amount of double-stranded DNA above the detection limit. The curve then plateaus in the third phase. The third phase is entered when the DNA is no longer being amplified, often due to a limiting reagent or exhaustion of the polymerase.

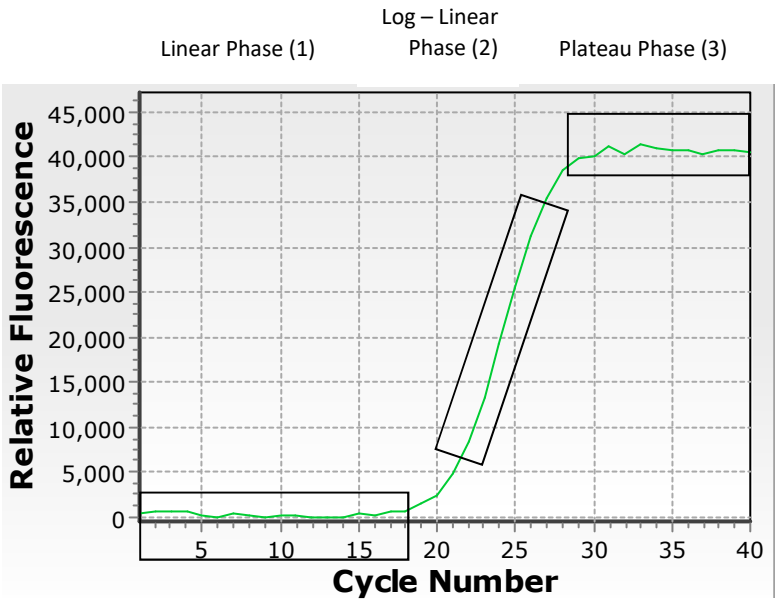


Figure 2.2: qPCR reaction showing the fluorescence at each cycle forming a sigmoid curve with three phases. The linear phase (phase 1), the log-linear phase (phase 2) and the plateau phase (phase 3).

A dissociation curve analysis, shown in figure 2.3, can be completed at the end of the qPCR to confirm that the fluorescence signal is generated from the amplified target DNA and not from nonspecific PCR products (Smith and Osborn, 2009). If the qPCR reaction has generated a specific product of the target DNA, then the DNA will denature at a specific temperature, resulting in a steep decrease in the level of fluorescence, as the dye is no longer bound to double-stranded DNA.

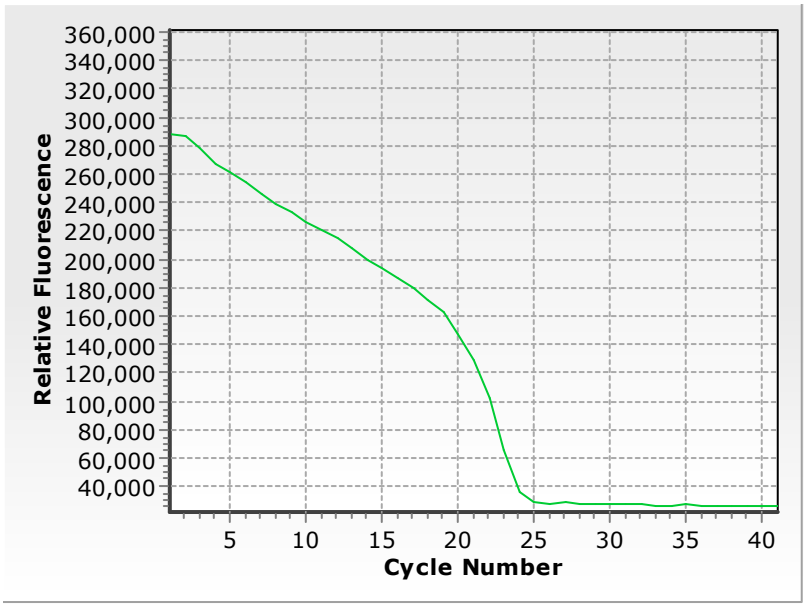


Figure 2.3: Dissociation curve analysis showing the relative fluorescence against cycle number (temperature). The steep drop in fluorescence occurs when the product denatures.

Traditional PCR can detect whether a specific target DNA is present within a sample. However, this is not quantitative as practically the end-point concentration of DNA is not directly proportional to the amount of the original template DNA due to inherent biases and limitations of the process (Smith and Osborn, 2009, Kim *et al.,* 2013). On the other hand, qPCR allows the quantification of the specific target. However, the specificity of both processes relies upon the design of the primers (Smith and Osborn, 2009). Theoretically the efficiency of the qPCR reaction should be 100%, meaning that the PCR product is doubling at each cycle. Practically, efficiency is dependent upon primer binding and successful completion of elongation. Primers may bind to the wrong area if specificity requirements are not met, and can form extra non-specific product called primer-dimers. The sensitivity of the reaction is highly dependent upon the efficiency of the reaction.

Technical advances in PCR and qPCR amplification and the falling cost of the methodology has enabled the expansion of the application of this technique from phylogeography to the quantification of diets (Andrew *et al.,* 2013). For example, King *et al.,* (2008) reviewed recent advances and issues of molecular analysis of predation using PCR amplification of prey remains with samples from faeces and the digestive system. Alternatively, qPCR has been used as a tool to detect and quantify the occurrence of cryptic species in soil microbiology (Campos-Herrera *et al.,* 2011a, 2011b). Cryptic species are species that are not culturable but may be ecologically significant, the detection of which is being improved by molecular methods. The technique of PCR has also been applied to the detection of protists. Hide *et al.,* (2003) offers a set of primers designed to specifically amplify *Blepharisma japonicum*, and Haentzch *et al.,* (2011) presents a multiplex PCR with specific PCR primers for *Paramecium sp.* These studies are mainly concerned with developing a fast and accurate approach of defining a ciliate community for the purposes of testing water quality or environmental indicators for soil samples. Whilst this work uses the similar approach of PCR and qPCR, the aim is to detect and quantify specific bacteria species in experimental microcosms.

Incorporating the bacterial community into a mechanistic understanding of microbial experiments remains a challenge. To the best of my knowledge PCR and qPCR as a technique has not been applied to the enumeration of species specific bacteria before in this context, and is a novel approach to resolving the bacterial assemblage within an experimental microcosm. PCR and qPCR as a technique have high repeatability and high throughput. PCR can determine the absence or presence of a specific species. qPCR allows the quantification of species specific bacteria and is highly-sensitive and allows accurate enumeration over several orders of magnitude. This would improve the accuracy of bacterial enumeration and enable the process to be completed rapidly.

The aim of this chapter is to develop a qPCR methodology and investigate its application. During this process the sensitivity and efficiency of the method was analysed and the reasons for limits of detection explored.

2.3 Methods

2.3.1 qPCR as a tool for enumerating bacteria

For the purposes of enumerating bacteria qPCR is used to monitor the amount of genomic DNA present in the samples. As each individual present has a single genome, the quantity of DNA is equal to the number of individuals present in the sample. To analyse samples with an unknown quantity of bacteria it is necessary to create a calibration curve from a known standard. Practically the calibration curve is dependent upon the efficiency and sensitivity of the reaction. This calibration curve can be constructed by conducting serial dilutions of a bacterial sample and calibrating the qPCR result with a count of the number of colony forming units determined by traditional methods.

In this case, ten-fold serial dilutions of each bacterial species were made to provide known numbers of cells for analysis from an initial concentration of 1 optical density at 600nm. For bacterial cultures optical density is calculated using light scattering, not absorbance. The amount of light scatter is directly proportional to the cell density and is an established protocol for monoculture samples (Monod, 1949). In this study optical density was used as an approximate starting point and were then validated through serial dilutions on an agar plate. The dilutions were then used in qPCR reactions to quantify the amount of DNA present. The mid-point of each sigmoid curve gives the quantification cycle (cQ) value – the number of PCR cycles needed to amplify to this level, which is proportional to the amount of template present (i.e. the number of cells present), see figure 2.2. The cQ values can then be plotted against the dilution factor to produce a calibration curve that allows unknown samples to be quantified.

2.3.2 Optimising the process

To optimise the PCR reaction and refine it for qPCR, certain reagents can be adjusted, including the polymerase and the primers. The optimisation is outlined below. Tests to use an expressed recombinant phusion polymerase were completed (Appendix A) to reduce cost and use a higher quality polymerase. However results were found to be variable. Therefore, to maintain constant conditions throughout the experiment a MyTaq^{TM} HS Mix (Bioline) was used.

Primer design

Primers for the bacterial species *Bacillus cereus* (ATCC 14579), *Pseuodomonas fluorescens SBW25* and *Serratia marcescens subsp.* (ATCC 13880) were designed using the software Primer-BLAST, see figure 2.4 (Altschul *et al.,* 1990, Ye *et al.,* 2012). This software was programmed so that candidate primer pairs met certain criteria, including specificity requirements. Primer-BLAST searches for primers against the target template, and can check that there are no mismatches against non-target species. Bases that don't match alter the annealing temperature, thus defining the specificity. The primers for *B. cereus* and *P. fluorescens* were designed for a glycolysis gene; glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Meanwhile primers for *S. marcescens* were designed using the gene pyruvate kinase due to the variability in the GAPDH gene between strains. These genes were chosen as they are generally highly conserved and this enables the specific detection of multiple subspecies for each species. Once Primer-BLAST defined a number of candidate primer pairs, the selection could then be completed manually. Multiple primer pairs were designed and selected to test sensitivity and limit cross-reactivity. Dieffenbach *et al.,* (1993) defines specificity as the frequency of mis-priming events and explains that primers with poor specificity are likely to produce undesirable amplicons. Additionally, care must be taken that the primer pairs are not complementary, otherwise a primer-dimer product could be formed, whereby the PCR product obtained is the amplification of the primers themselves (Dieffenbach *et al.,* 1993).

It is desirable that the primers have:

- a 50%-60% GC (guanosine-cytidine triphosphates) content; these form stronger bonds in the amplified DNA sequence
- Melt temperature around 60°C provides a sufficient opportunity for annealing. Melt temperatures should be similar for both primers within a pair.
- Low self-complementarity and complementarity, so as not to bind in the wrong region
- A sequence with final bases of G or C, as these bind more strongly to the DNA strand.
- A product size of less than 200bp; this enables a faster cycle program.

For each bacteria species two or three primer pairs were selected (shown in table 2.2). These primer pairs were selected based on the above requirements. *S. marcescens* had fewer primer options due to the high specificity requirements of design, so for this species only two primer pairs were tested. Testing multiple primer pairs provides a choice, if a pair is found to be inefficient or not suitable for PCR due to variability or cross-reactivity, then another primer pair can be selected. Primer pairs selected for testing were also chosen to represent different sections of the gene DNA sequence. This ensures that if one section of the DNA sequence is not particularly suitable for PCR, then it should only affect one primer pair.

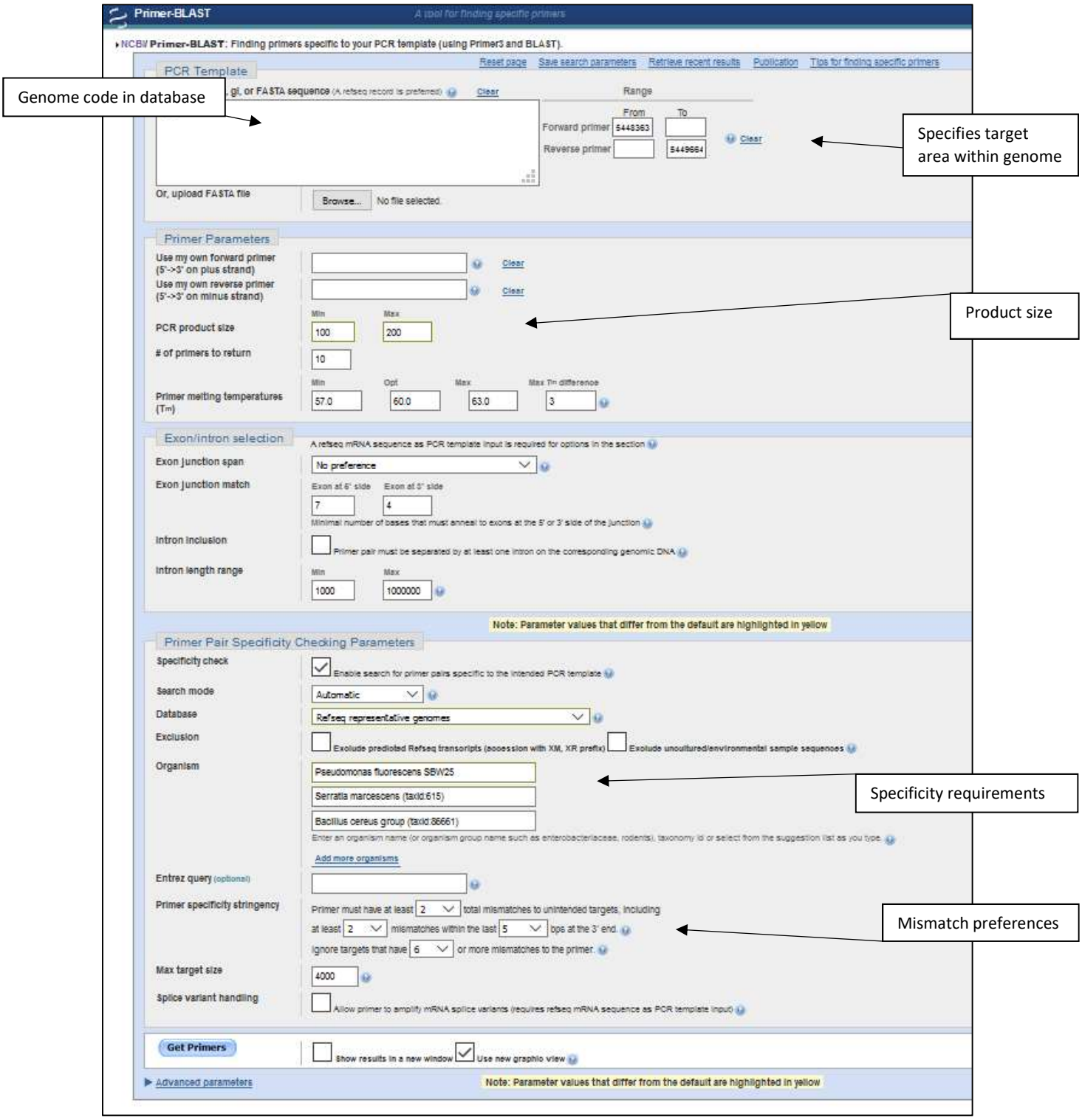


Figure 2.4: The Primer-BLAST interface. Highlighted are the parameters changed to generate the primers. http://www.ncbi.nlm.nih.gov/tools/primer-blast/

Primer validation and specificity

To ensure the primers targeted the correct species without cross matching with other DNA sequences they were tested by PCR. PCR was completed using a Bio-Rad T100™ gradient thermal cycler. The following PCR program was used to confirm primer specificity and ensure the correct amplification of DNA and no amplification when non-target species are present. 5-minute denaturation at 96°C, followed by 35 cycles (20 seconds denaturation at 96°C, 20 seconds annealing at 55°C, 20 seconds elongation at 72°C) and a final elongation step at 72°C for 4 minutes. Products were checked by gel electrophoreses. This PCR procedure also tested that amplification occurred under this program efficiently and was utilised to optimise the procedure for the qPCR program. In order to include a control primer pair in the validations, the primer pairs shown in table 2.1 were used. These primer pairs were designed previously and tested for PCR.

Table 2.1: The control primers for *B. cereus, P. fluorescens* and *S. marcescens,* previously designed and tested for PCR. Primer code, sequence and product length is shown.

Code	**Primer Pair**	**F/R**	**Sequence 5' -> 3'**	**Product Length**
BC-F	BC450	F	GCTCTTCACCGGGAATGCT	450
BC-R		R	AGTTTTGAGGCTGCGCTTTC	
PF-F	PF798	F	ACACCGTTCATGGCACATTC	798
PF-R		R	CCCACTGTTGTCGTACCA	
SM-F	SM369	F	CGTCATTTACGCCGCTTCTG	369
SM-R		R	ATCGGTAAAGAAGCCCGTC	

Electrophoresis

Agarose gel electrophoresis was used to analyse the PCR products. The gel was made with 1.5% Agarose (Fisher Bioreagents) with TE buffer and stained with 0.1mg/ml Ethidium Bromide. The molecular weight marker used was 100bp DNA ladder (HyperLadder™ 100bp, Bioline). Gels were run at 80V for 20 – 30 minutes and were visualised on a gel doc (UVP GelDoc-IT Imager).

Primer sensitivity and efficiency

It was noted that primer pairs could have different levels of sensitivity and efficiency. Where efficiency or sensitivity was deemed to be low in the final calibration curve, the alternative primer pair was tested. Additionally, the reasons for low efficiency or sensitivity were investigated.

2.4 Results

The primers designed and selected using Primer-BLAST are shown in table 2.2. These primers were designed to specificity requirements that ensured no mis-matches between the species *B. cereus, P. fluorescens* and *S. marcescens.* The primer products were also designed to be less than 200bp to be suitable for qPCR.

Table 2.2: Primer pairs designed by Primer-BLAST for *B. cereus* (BC), *P. fluorescens* (PF) and *S. marcescens* (SM), showing code, sequence and product length.

Code	**Primer Pair**	**F/R**	**Sequence 5' -> 3'**	**Product Length**
BC1-F	BC1	F	GGAACAAACCCTCTCCCTCG	96
BC1-R		R	CGCGTAGTAGACCTAGCAGC	
BC5-F	BC5	F	ATCATTCCGCGTTTTACGCC	109
BC5-R		R	ACGATGCAGCTAACCACAAC	
BC9-F	BC9	F	TGGATTTCCCTCCGACTACT	108
BC9-R		R	CGAGGGAGAGGGTTTGTTCC	
PF3-F	PF3	F	GTATACCCAAGGCTACCGCC	143
PF3-R		R	TCAGGCTTTCCTGATCGTGG	
PF4-F	PF4	F	ACATGATCCCGAGCAAGACC	135
PF4-R		R	TCAACTGCACAGTGAGGTCC	
PF8-F	PF8	F	CTACGGCGTCAACCATGACA	149
PF8-R		R	TGTAGGCGTGAATCGTGGTC	
SM2-F	SM2	F	CGATTTCGCGCACTTTGTCA	167
SM2-R		R	CAGACGGCTCAGAAGAACCA	
SM3-F	SM3	F	CGTGGGAGAAGTTAAGCCGA	120
SM3-R		R	CCAGACGGCTCAGAAGAACC	

2.4.1 Primer validation

The primer pairs for each species were tested with the target species, no template controls and non-target species. The results can be seen in figure 2.5, displaying the agarose gel electrophoresis. For each species the 100bp molecular weight marker is shown in the lane marked M. The control products (table 2.1) in lane 1 were ~500bp for *B. cereus* and ~800bp for *P. fluorescens* and *S. marcescens*. For *B. cereus* and *P. fluorescens* in lanes 2, 3 and 4 are the primer pairs from table 2.2 with the targeted species. As expected, all primer pairs worked and produced a band of ~100bp. The brightest band for *B. cereus* from the optimised primers is BC9 and for *P. fluorescens* is PF8. For *S. marcescens* the designed primers from table 2.2 are in lanes 2 and 3 with the target species. Both primer pairs produced a product of the right size. As there are no product bands formed in the no template controls or in the presence of the non-target bacterial species it is clear the primers specificity requirements have been met for all primer pairs. Primer pairs BC9, PF8 and SM2 were selected as these products were judged to produce the brightest and clearest bands and did not show evidence of cross-reactivity.

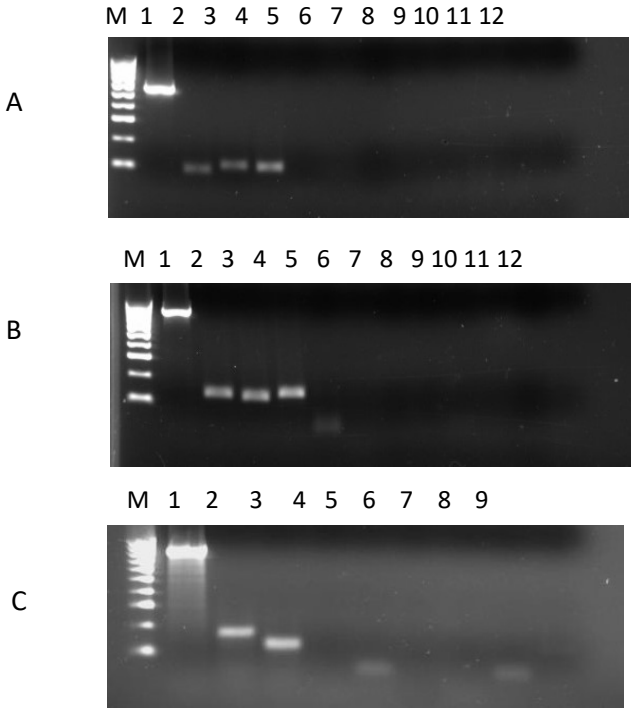


Figure 2.5. The *B. cereus* control primer, and three designed primer pairs (Bc1, 5, 9) are shown in A. *The P. fluorescens* control primer, and three designed primer pairs (Pf 3, 4, 8) are shown in B. The *S. marcescens* control primer, and two designed primer pairs (Sm2, 3) are shown in C. For each gel the molecular weight marker is in the lane marked as M. The respective control primers are in lane 1. For *B. cereus* and *P. fluorescens* the three designed primers are in lanes 2, 3 and 4 with the desired target species. For *S. marcescens* the two designed primers are in lanes 2 and 3 with *S. marcescens* present. No template controls for each primer pair are in lanes 5, 6, 7, 8 for *B. cereus* and *P. fluorescens,* and in lanes 4, 5, 6 for *S. marcescens.* For *B. cereus* and *P*. fluorescens lanes 9, 10, 11 and 12 represent the primers with the non-target species. For *S. marcescens* the primers were tested with the non-target species in lanes 7, 8, and 9.

The dissociation curves for the primer pairs BC9, PF8 and SM2 are shown in figure 2.6 alongside the no template controls. The steep drop in fluorescence shows a specific product was formed and denatured at a specific temperature (cycle number). This shows that the primers formed specific products when used for qPCR.

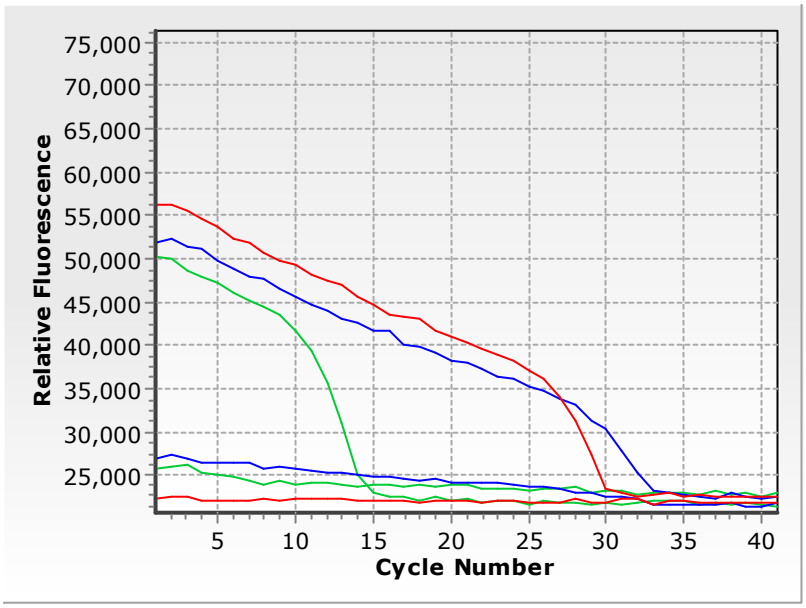


Figure 2.6: The dissociation curve analysis for primer pairs BC9 (green), PF8 (blue) and SM2 (red). The samples showing low starting fluorescence and a slight gradual decline are the no template controls. The samples showing a steep drop in fluorescence are the samples representing the species. The primers for *B. cereus* formed a steep drop in fluorescence at 14 cycles, *P. fluorescens* at 32 cycles and *S. marcescens* at 30 cycles.

2.4.2 Calibration curves

To calibrate the fluorescence curves produced by qPCR, ten-fold dilutions of each bacteria species from an initial culture at 1 optical density were created. The dilutions were created in a microtiter plate using 20µl of cells and 180µl of dH_2O. The first dilution set was carried out in the first row, left to right. Dilution sets were then created from each original serial dilution vertically, top to bottom, to validate the first serial dilutions, shown in figure 2.7. 2µl of each serial dilution was plated on agar plates in the same pattern and grown overnight at 22°C. If serial dilutions were completed correctly, diagonal spots on the plate should contain equal numbers of colony forming units. The number of colony forming units (cfu) for each dilution represented the number of cells initially present in each dilution under the assumption that one colony is formed from one bacterial cell. These plates are shown in figures 2.8**.**

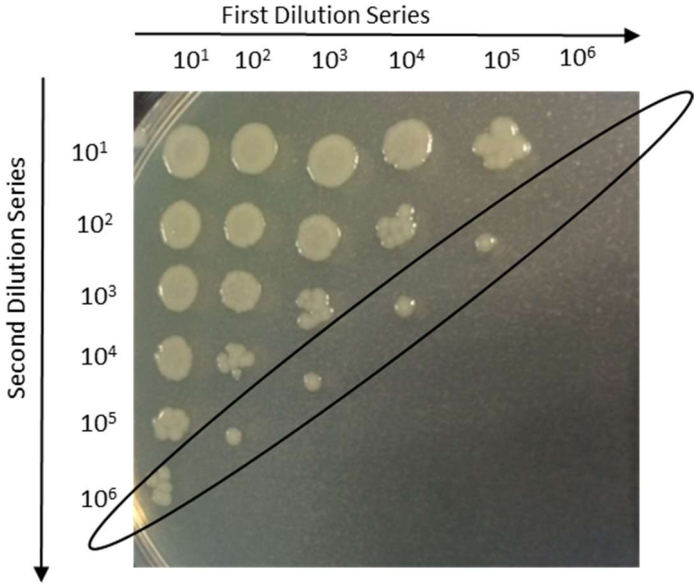


Figure 2.7: Schematic of the dilution series on an agar plate. The first dilution series is conducted in the first row from left to right. Each dilution point from this series acts as the first dilution point vertically. Dilutions should therefore be equal horizontally. This enables the validation of the dilutions. In this example the 10^6 dilutions in the first column and row are revealed to be different to the other diagonal 10^6 dilutions, therefore the dilution can be considered false.

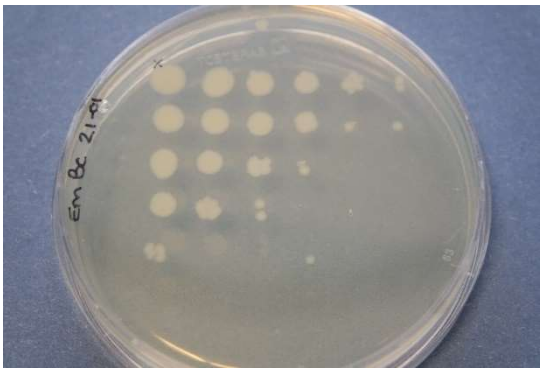
The count of bacteria per µl can be calculated from the serial dilution using Equation 1.

Equation 1: $$No.of\ cfu\ x\ dilution\ factor\ x\ \frac{1}{2} = no.of\ individuals\ per\ 1\mu l$$

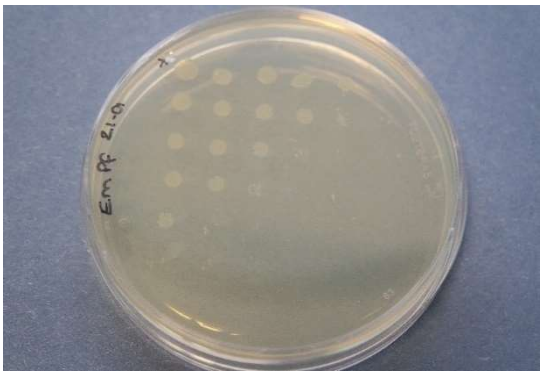
It can be seen in figure 2.8, that for a 1OD sample of *P. fluorescens* one cell is present in 2µl of a dilution of 1/1,000,000. This means that in a 1µl sample at 1OD there is approximately 500,000 cells, as shown in Equation 2. Likewise, for *S. marcescens* it was estimated that in a 1µl sample at 1OD there was approximately 700,000 cells and 50,000 for *B. cereus.*

Equation 2: $$1\ x\ 1{,}000{,}000\ x\ \frac{1}{2} =\ 500{,}000$$

A



B



C

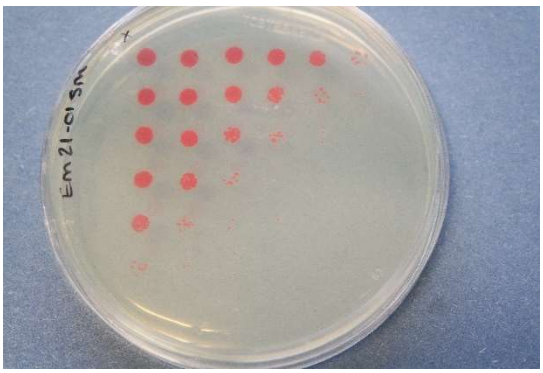
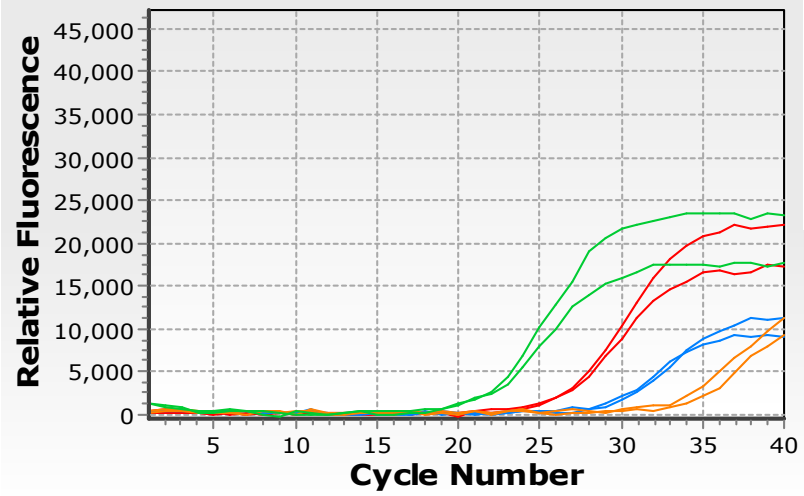


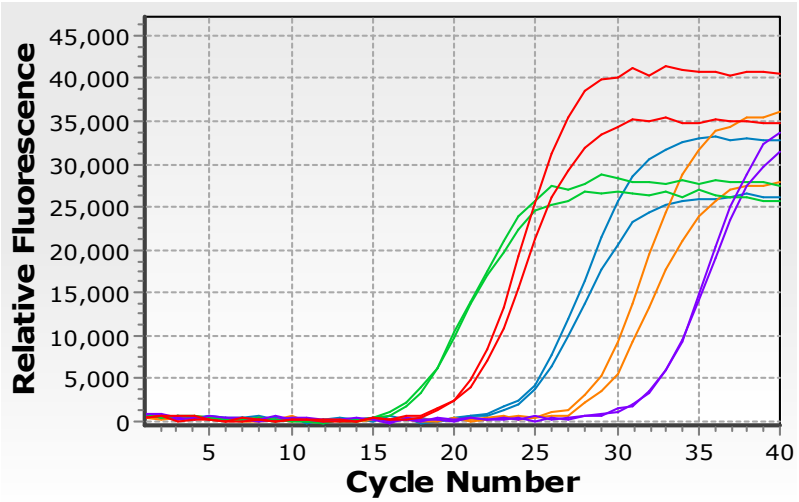
Figure 2.8: Ten-fold serial dilutions of *B. cereus* (A)*, P. fluorescens* (B) and *S. marcescens* (C) with an initial concentration of 1OD at 600nm. The sample was first diluted in the top row, left to right. Each of these dilutions was then diluted further from top to bottom to validate the first dilution set.

qPCR reactions were conducted in duplicate using the same ten-fold serial dilutions with the selected primer pairs. This formed the staggered sigmoid curves shown in figure 2.9. The qPCR reactions with fewer cells initially as a result of the serial dilution required more cycles to reach the cQ. Therefore, the higher the cQ the fewer cells present in the sample. With the ten-fold dilutions it was expected that the cQ for each dilution should be ~3.5 cycles apart.

A



B



C

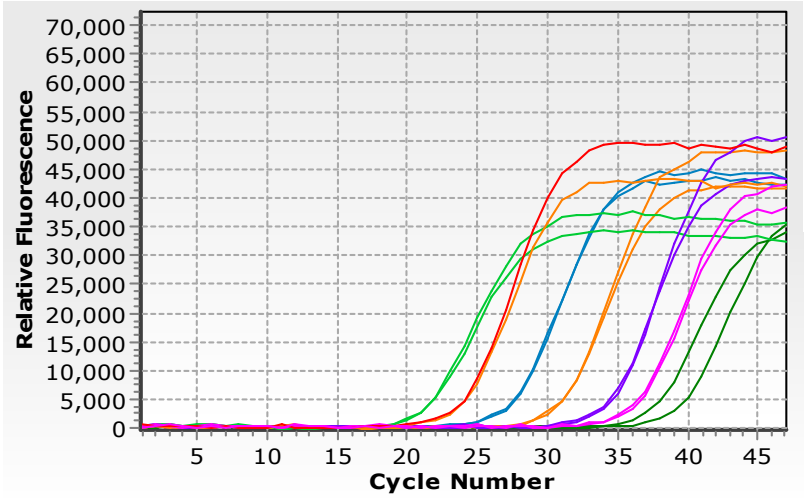
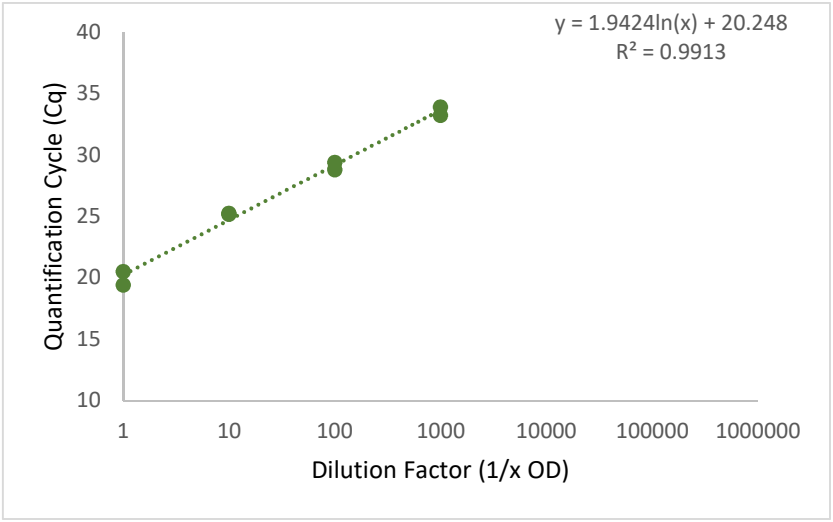


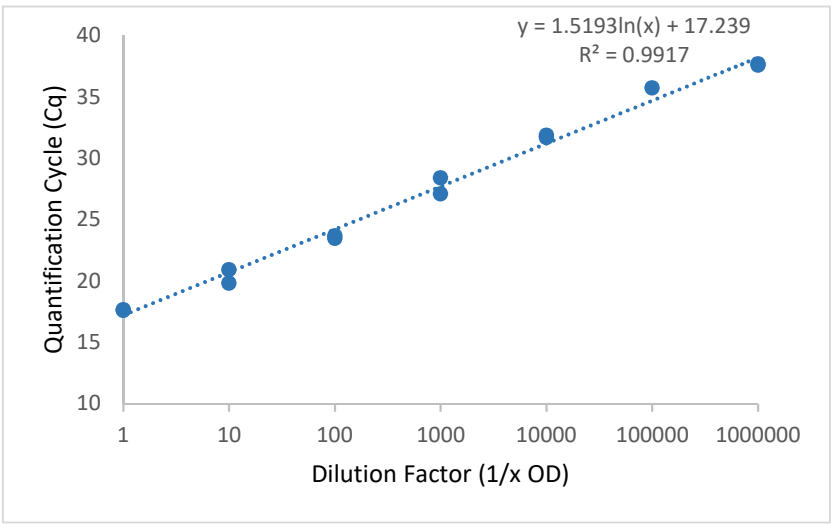
Figure 2.9: qPCR amplifications showing fluorescence of double-stranded DNA amplicon against the number of cycles for *B. cereus* (A), *P. fluorescens* (B) and *S. marcescens* (C). Primer pairs BC9, PF8 and SM2 were used. Light green is the initial concentration at 1OD. The red curves show the 1/10 dilution, blue is 1/100, orange 1/100, purple is 1/1000, pink 1/10,000 and dark green 1/1,000,000.

The calibration curves shown in figure 2.10 show the cQ values plotted against the dilution factor. Repeat qPCR reactions for each dilution produced very similar cQ values. *P. fluorescens* and *S. marcescens* show good sensitivity with detection at 1/1,000,000 dilution, which represents a single cell. Meanwhile *B. cereus* only showed detection to a concentration of 1/1000. Furthermore, considering the slope of each calibration, the reaction for *B. cereus* is the least efficient.

A



B



C

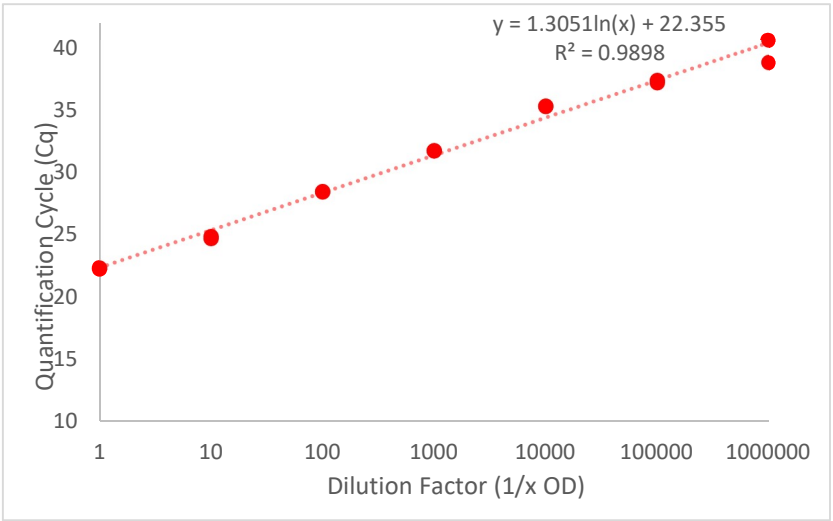


Figure 2.10 Calibration Curves plotting the quantification cycle value against dilution factor for *B. cereus* (A), *P. fluorescens* (B) *and S. marcescens* (C) using primers pairs BC9, PF8 and SM2. Reactions were completed in duplicate, which may overlap.

Using these curves (fig 2.10) provides equations which can be used to convert cQ values to dilution factor. Using the plate counts (fig 2.8) of the serial dilutions the dilution factor can then be converted to the number of cells per 2µl. This two-step conversion is shown below in equations 3-8 for each bacterial species.

B. cereus:

Equation 3: $DF = e^{\frac{(cQ-20.248)}{1.9424}}$

Equation 4: $\mathrm{c} = 100{,}000 \times DF^{-1}$

P. fluorescens:

Equation 5: $DF = e^{\frac{(cQ-1\ .239)}{1.5193}}$

Equation 6: $\mathrm{c} = 1{,}000{,}000 \times DF^{-1}$

S. marcescens:

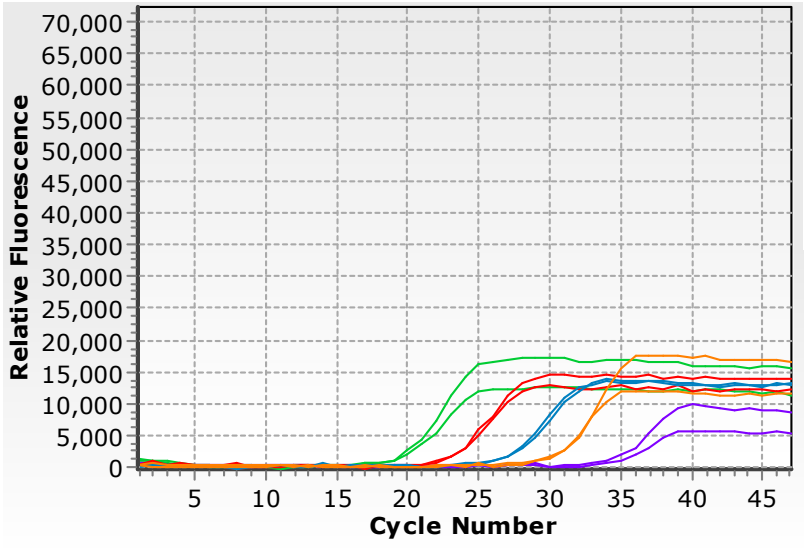
Equation 7: $DF = e^{\frac{(cQ-2\ .355)}{1.3051}}$

Equation 8: $\mathrm{c} = 1{,}400{,}000 \times DF^{-1}$

2.4.3 Investigating sensitivity and efficiency

Testing a different *B. cereus* primer pair reveals the same trend in sensitivity, although the efficiency of this primer pair is higher (fig 2.11). This suggests that the sensitivity may not be a direct outcome due to the primer design. To further investigate the reasons why the *B. cereus* reaction may be less sensitive but still efficient, *B. cereus* was examined microscopically (fig 2.12). *B. cereus* was cultured in LB media for 24 hours. It can be seen that *B. cereus* readily forms chains of individuals and therefore is not homogenously dispersed. This means that at low density the detection of *B. cereus* becomes stochastic due to high error in sampling a representative density of *B. cereus.* Figure 2.12 shows that these chains of individuals can be disrupted and broken apart by sonication. In figure 2.12A chains of *B. cereus* individuals can be seen with few individual cells. However, after sonication, shown in figure 2.12B these chains of *B. cereus* are broken apart into individual cells. Although there are still a few chains formed by two cells, so sonication does not completely separate all individuals.

A



B

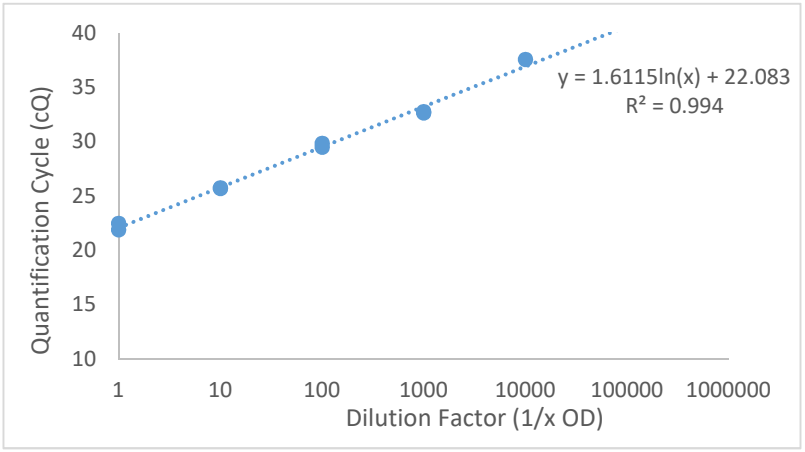


Figure 2.11. qPCR amplification of *B. cereus* using primer pair BC1 (A). The red curves show the 1/10 dilution, blue is 1/100, orange 1/100 and purple is 1/1000 in duplicate. The resulting calibration curve plotting the quantification cycle value against dilution factor (B) Duplicates may overlap.

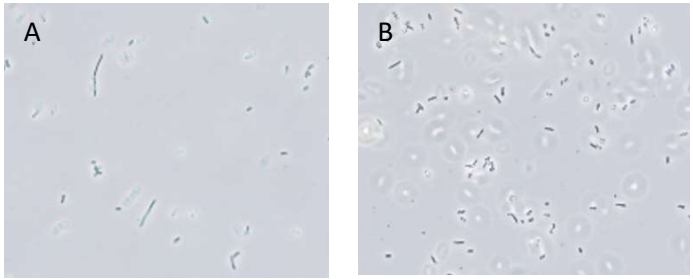


Figure 2.12. Image of *B. cereus* in LB media readily forming chains of individuals (A). *B. cereus* in LB media after 10 minutes of sonication (B). Chains are generally broken by sonication and create a more homogenous distribution of *B. cereus.*

2.4.4 The 'gritty media problem'

The qPCR validations were completed with bacteria grown in LB media. Yet LB media is unsuitable for the cultivation of protists. Microcosm experiments use many types of media, often formed from the suspension of organic matter (for an overview see Altermatt *et al.,* 2015, Supplementary Information, Section 1.2). While the components of LB media are chemically well-defined and fully soluble, medias commonly used are less well-defined and often contain particulate matter. In this study, media was constructed by suspending 0.5g/l powdered chlorella (Naturya Organic Chlorella) in mineral water, which acted as an organic nutrient and carbon source. Hereafter this media is referred to as chlorella media. It was found that the sensitivity of the qPCR reaction declined when applied to a media containing particulate matter such as chlorella media. Microscopic imaging showed that the particulate matter in the media encouraged aggregation of the bacteria and biofilm formation (fig. 2.13). In figure 2.13, a chain of *B. cereus,* concentrated area of *P. fluorescens,* and three dense chains of *S. marcescens* are shown circled (panels A, B and C respectively). It is known that *B. cereus* forms chains of individuals in LB media from figure 2.12A. However, it can be seen in figure 2.13D and E that *P. fluorescens* and *S. marcescens* are individually dispersed in LB media and were highly motile. Therefore, it is suggested that the presence of particulate matter in the media encourages the aggregation of bacteria.

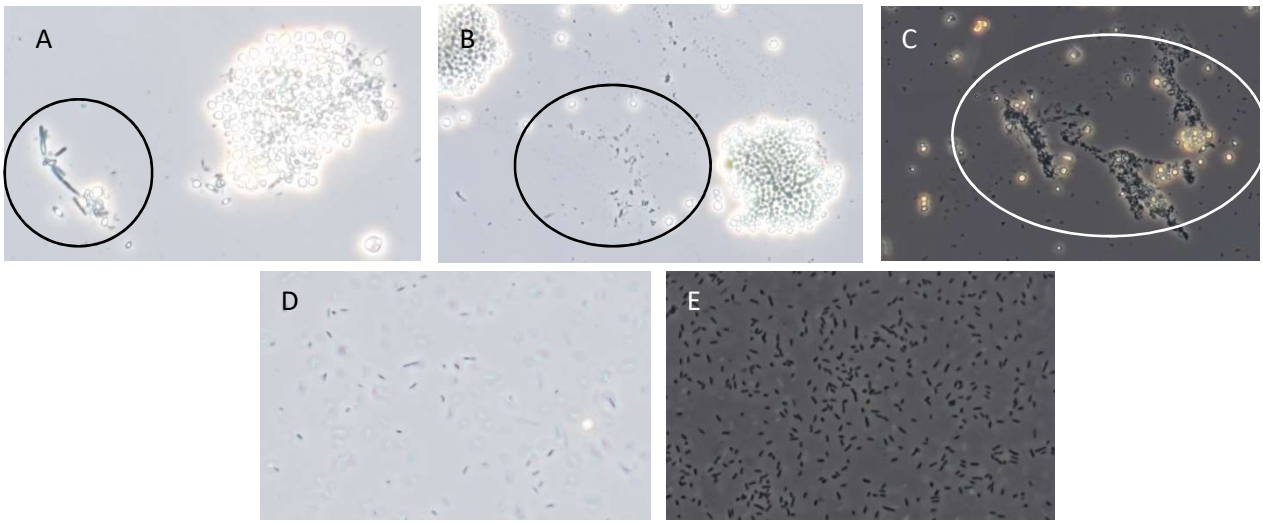


Figure 2.13: Microscopic images of *B. cereus* (A), *P. fluorescens* (B) and *S. marcescens* (C) in Chlorella media and motile *P. fluorescens* (D) and *S. marcescens* (E) in LB media. All three bacterial species form aggregates around particulate matter within the chlorella media (circled).

By sonicating the samples for ten minutes these groups of bacteria can be detached from the clumps within the media and from themselves to create a more homogenous distribution (fig 2.14). Figure 2.14 shows particulates within the cultures that are 'clean' of bacteria following sonication which disrupted the bacterial aggregates. In figure 2.14C, it can be seen that whilst sonication has separated *S. marcescens* from particulate matter, *S. marcescens* individuals are still part of a cluster.

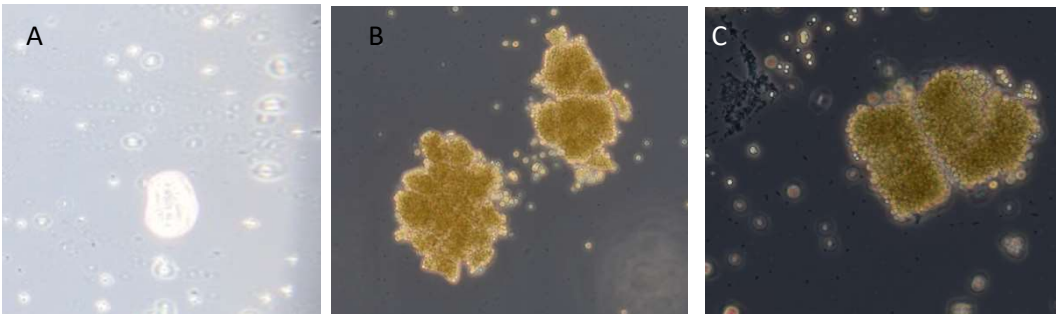


Figure 2.14: *B. cereus* (A), *P. fluorescens* (B) and *S. marcescens* (C) in Chlorella media after 10 minutes of sonication. Sonication generally disrupts the aggregation of bacteria around particulate matter within the media, separating and distributing bacteria cells individually.

To examine the response of bacterial aggregation in the presence of a more complex community a predator, *Paramecium caudatum,* was added to the cultures. The microscopic images of these cultures for *P. fluorescens* and *S. marcescens* are shown in figure 2.15. The culture for *B. cereus* is not shown as the presence of a predator caused there to be very little *B. cereus* present. *P. fluorescens* and *S. marcescens* develop a concentrated population around particulate matter and appear to be embedded in a biofilm. It was observed that in the presence of a predator the formation of this biofilm is much more frequent, suggesting that the presence of a predator triggers this response in the bacteria.

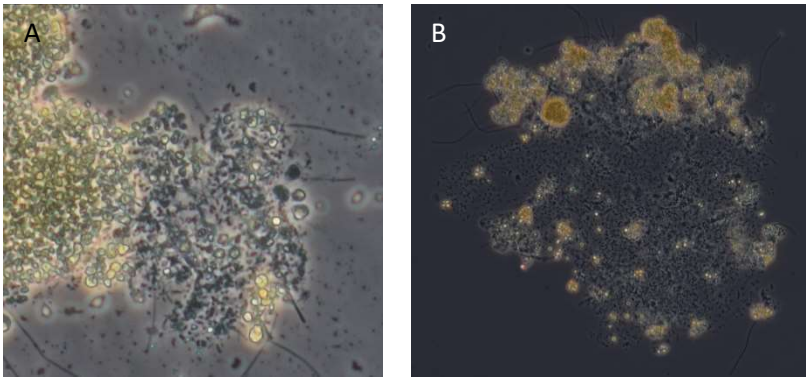


Figure 2.15: *P. fluorescens* (A) and *S. marcescens* (B) in Chlorella media with the bacterivorous protist, *Paramecium caudatum*. Both bacteria have aggregated around particulate matter and have formed a biofilm.

2.4.5 Freezing samples

Using qPCR as a tool to enumerate bacteria species in ecological microcosm experiments could be made more accessible for ecologists if freezing samples were a possibility. This would enable samples to be collected from microcosms, frozen, and either tested at a later, more suitable date or sent to another facility for the qPCR process. This could help ecologists manage the time aspect of using qPCR reactions for quantification and also offer access to ecologists who do not have experience of qPCR or the equipment to use qPCR. However, the process of freezing must be proven to not interfere with the results, either through DNA degradation or another mechanism. To investigate the possibility of freezing samples, monocultures of bacteria were created, grown at 26°C and sampled 48 hours after inoculation and frozen and sampled after 3 days and 6 days of freezing.

Samples were grown for 48 hours before testing and freezing to ensure bacterial cultures were dense. To ensure the detection was unchanged with different concentrations of bacteria qPCR reactions were conducted at full concentration and at a 1/100 dilution. The results summarised in table 2.3, show that there is variation in the cQ value dependent upon whether the sample is fresh or frozen, and there is a change in the cQ value with the length of freezing. Whether the sample is fresh or frozen, and the length of time frozen has an effect on the average cQ value that is proportional to a ten-fold change in number of cells. These results indicate that there are complex processes acting upon the DNA during the freezing process and more work is needed in order to set a standard freezing procedure.

Table 2.3: Average quantification cycle value for fresh, and frozen samples of *B. cereus, P. fluorescens* and *S. marcescens* at full concentration after 24 hours of growth and 1/100 dilution. Samples were tested after 3 days and 6 days of freezing. The variation in cQ values shows the action of processes altering the amount of DNA.

	Average cQ Value		
Sample	**Fresh**	**Frozen for 3 days**	**Frozen for 6 days**
B. cereus	32.08	23.55	28.83
B. cereus 1/100	36.41	29.01	35.8
P. fluorescens	22.89	17.29	20.78
P. fluorescens 1/100	29.66	23.95	27.93
S. marcescens	32.29	28.78	30.69
S. marcescens 1/100	40.6	33.99	37.59

2.4.6 Detection of rare species

In experimental microcosms when searching for a rare species it is common practise to either, search a larger volume of the microcosm, search 10% of the microcosm, or even search the whole microcosm. However, doing this for bacteria would not be possible due to adding large volumes. However, to detect elusive species it may be possible to centrifuge a larger sample size, remove the supernatant and resuspend the pellet. While this may not provide an accurate estimate of the species abundance due to the size of handling errors during this process this may enable the detection of the species within a larger volume by condensing the sample size. This was tested using 1ml of chlorella media, when *B. cereus* was suspected to be a rare, if not extinct species. Unfortunately, centrifuging the sample, not only concentrated the bacteria into a pellet, but also the solid component of the media. The solid component of the media interfered with the qPCR reaction and prevented the qPCR reactions from successfully amplifying a product (data not shown).

2.5 Discussion

2.5.1 qPCR, and its limitations

The development of a qPCR procedure and the production of these calibration curves demonstrates the development of a technique which enables the quantification of unknown samples of specific species. This will therefore allow the monitoring of the bacterial community. This will provide a greater resolution of the food web and enable the investigation of the effects of bacterial dynamics upon experimental communities. The calibration curves show that the cQ very strongly correlates with the concentration of bacteria and can be used to accurately quantify the amount of species-specific bacteria present.

For *P. fluorescens* and *S. marcescens*, the validation showed sensitivity down to the presence of a single cell in the assay. The qPCR reaction for *B. cereus* was less efficient, and much less sensitive. Whilst investigating the reasons for this, it was found that *B. cereus* typically forms chains (see fig. 2.11A). Other species of *Bacillus* have been reported to form chains such as *B. funiculus* (Ajithkumar *et al.,* 2001, Ajithkumar *et al.,* 2002), *B. mycoides* (Di Franco *et al.,* 2002) and *B. macauensis* (Zhang *et al.,* 2006). This characteristic of *B. cereus* has an impact upon the sensitivity of the qPCR reaction and causes the detection of *B. cereus* at low density to become inconsistent, as shown in figures 2.9A, 2.10. The heterogeneity of the *B. cereus* distribution interferes with sampling a representative sample and can often mean that either a chain of many individuals or no individuals are sampled. This issue can be partly overcome by sonicating the samples to disrupt the chains, as evidenced in figure 2.11. Additionally, it was found that in 'gritty media' the bacteria form groups and biofilms around particulate matter in the media which can make detection less reliable. Sonication has also been used to somewhat resolve these issues (fig. 2.12). Sonication has been incorporated in some studies as part of the preparation of samples for flow cytometry. For example, Limberger and Wickham (2011) sonicated their samples of algae and bacteria before enumeration via flow cytometry to ensure the disaggregation of clumps. Haaber *et al.,* (2016) reports that sonication improves the accuracy of cell counts from plate counts of *Staphylococcus aureus*, which also readily forms aggregates.

Whilst investigating the effects of sonication upon bacteria (figs. 2.12 and 2.13) it was noted that there were multiple morphotypes of each bacterial species, and these morphotypes responded to the media composition. In LB media, whilst *B. cereus* readily formed chains, *P. fluorescens* and *S. marcescens* remained motile (fig. 2.12D, E). However, in Chlorella media, the presence of particulate matter in the media, encouraged the formation of groups and some biofilms (fig. 2.12). To investigate the selective pressures encouraging group and biofilm formation, a predator, *Paramecium caudatum*¸ was added to the monocultures. It was found that the morphotypes switched further, with many more individuals producing a biofilm (fig. 2.14). The appearance of the biofilm changed to embed the bacteria within the biofilm, potentially creating a refuge for the bacteria. This shift was clearly related to predatory pressure, however further investigation is needed to test this and assess the mechanism for this response.

The ability to freeze samples for qPCR on a later date or delivery to a qPCR lab would make this approach much more accessible. However, preliminary tests suggested that there were processes acting upon the frozen DNA sample, by altering the cQ value (table 2.3). Ultimately this impacts the reliability of the estimate for bacterial abundance. The processes influencing this could be linked to DNA degradation.

Further investigation is required into the detection of extremely rare species. Centrifuging a volume of the sample aims to condense rare species into a smaller volume, enabling their detection. However, this process caused interference and ultimately failure of the qPCR reactions. Experimenting with the effects of filtering to remove particulate matter, or alternative media could resolve this issue.

One often cited limitation of qPCR is its inability to discriminate between live and dead cells (Alvarez *et al.,* 2013). This causes an overestimation of the abundance of live cells present. Studies have proposed that treating samples with propidium monoazide prior to DNA extraction, removes dead cells from detection by qPCR (Nocker *et al.,* 2009, Fittipaldi *et al.,* 2012, Alvarez *et al.,* 2013). This method distinguishes dead or live cells based upon membrane integrity, as propidium monoazide enters cells with compromised membranes and stops the DNA from interacting with the qPCR reaction. The detection of dead cells was not encountered in these circumstances, likely due to the fast rate at which energy was recycled.

2.5.2 Comparison to other methods

Traditionally the enumeration of bacteria has relied upon counts from serial dilutions. However, as mentioned this method is time and labour intensive. qPCR provides a rapid approach to accurately quantifying bacteria in experimental microcosms. Where the investigation of bacteria was limited due to time constraints, there was a trade-off between the depth of investigation and time required for the method. The development of qPCR eases this trade-off between time and scale of study and enables a more thorough investigation of the bacterial community. Furthermore, as qPCR detects and defines the presence of specific species, providing specificity requirements are met, there can be high confidence in the accurate identification of the bacteria. Meanwhile traditional culture methods rely upon morphological identification of species (Altermatt *et al.,* 2015). However, even with a well-defined morphologically distinct starting community, later identification is not always possible (Cochran-Stafira and von Ende, 1998, Jiang and Morin, 2004). Another criticism of traditional culture methods is that the conditions under culturing may not allow representative growth (Franklin *et al.,* 2001) and thus do not detect cryptic species. On the other hand, qPCR could potentially detect and quantify cryptic species, if primers were suitably designed for the species.

The tendency for *B. cereus* to form chains has implications for other methods of detection, such as serial dilutions. To obtain a count from a serial dilution, it is assumed that one colony forming unit has grown from one cell. However, it is possible and perhaps likely, that a colony forming unit has grown from a chain of cells rather than just one. This suggests that serial dilutions may not provide an accurate absolute count of certain bacterial species and has also been noted by Hazan *et al.,* (2012). Additionally, Haaber *et al.,* (2016) reports that bacterial aggregation affects optical density measurements. Some studies have used flow cytometry to attain information on the bacterial community (Limberger and Wickham, 2011). Flow cytometry is a fast technique which can provide a broad range of information at the single-cell scale (Hammes and Egli, 2010). One disadvantage is that samples require filtering, to ensure the pathways do not become clogged. Samples with protists in and particulate matter are extremely likely to clog the flow cytometer. Therefore, this method is not suitable for communities such as those here. A key issue with flow cytometry is separating the signals from background noise and abiotic particles. During preliminary investigations, testing flow

cytometry for bacterial detection and enumeration, it was found that the chlorophyll in chlorella media fluoresced and obscured any signal from bacterial cells.

2.5.3 Potential future applications of this qPCR approach

The development of a qPCR approach to quantifying bacteria in experimental microcosms qPCR could allow the investigation of a broad range of ecological questions. In addition, using qPCR could be applied to revisit previous studies where bacterial dynamics have been inferred as a potential cause for observed dynamics. This could improve our mechanistic understanding of species interactions and community response. For example, some studies that have investigated the bacterial community, have focussed upon the implications for competition and coexistence at higher trophic levels (Balciunas and Lawler, 1995, Fox and Smith, 1997, Jiang and Morin, 2004). These papers consider grazing efficiency of bacterivores and resource partitioning of the bacteria, yet cannot be specific regarding bacteria species due to methodological limitations. Additionally, Rønn *et al.,* (2002) showed change in bacterial community composition in terrestrial soil systems as a result of protist grazing pressure and the nature of change was protist-species specific interactions. Yet the study could not be specific about bacterial species. Revisiting these studies using the qPCR approach would aid the investigation of bottom-up control and the underlying mechanisms.

Greater monitoring of the bottom trophic level through qPCR, also enables the investigation of community processes and structure in experimental microcosms. The addition of data for the bottom trophic level would be useful for studies researching trophic cascades, food chain length, community stability and response to perturbations. qPCR could also be used to investigate bacterial competition, and predator-prey dynamics with bacterivores. qPCR could also be used in conjunction with other methods as part of a comprehensive molecular toolbox. qPCR has been developed for taxon-specific quantitative analysis using 16S RNA (Bachetti De Gregoris *et al.,* 2011). The development of this assay was shown to be useful in monitoring the shifts in higher taxa within young marine biofilms (Bachetti De Gregoris *et al.,* 2011). Taxon-specific qPCR and species-specific qPCR could work in combination to investigate broad trends of functional groups and detailed dynamics of select species in larger-scale communities.

2.6 Conclusion

It has been shown that the qPCR approach can allow the rapid detection and enumeration of bacteria and can detect bacteria within sensitivity of a single cell. The development of this technique can dramatically improve the accuracy of monitoring of the bacterial community within microcosms. qPCR offers an exciting opportunity to consider bacterial dynamics and their interaction with the community in a much more thorough and accurate manner. However, limitations of the technique need to be acknowledged. Exploring these limitations has raised interesting microbiological questions worthy of further investigation in their own right. For example, considering the prey responses of bacteria under predation with complex morphotype and biofilm mechanisms. Plus, the effects of media constitution upon the experimental outcomes, and how media manipulates spatial heterogeneity, has been highlighted as a complex avenue for investigation. With technical advances making molecular methodologies more accessible, it seems it is time to update our experimental toolbox.

3 The implementation of the qPCR approach: the effects of temperature and community structure on species interactions

3.1 Introduction

Understanding community response to climatic change is a key challenge in ecology. Current predictions suggest that global temperatures will rise between 0.3°C and 4.8°C over the next 100 years (IPCC, 2013). Such climatic change is likely to have profound ecological consequences (see Walther *et al.,* 2002). These predicted changes in global temperature make understanding the impact of temperature change upon ecosystems a priority. Temperature directly controls species survival, and affects the metabolic requirements of an individual, thus influencing biological processes and ultimately affecting processes of resource competition and predation (Brown *et al*., 2004, Clements *et al*., 2013). Alterations to these population dynamics and species interactions can be a major determinant of species abundance and consequently community structure (Jiang and Kulczyki, 2004*).* However, our ecological understanding of the role of species interactions in the response to environmental change remains limited (Jiang and Kulczyki, 2004, Shurin *et al.,* 2012). Therefore, in order to successfully model ecosystem change it is critical to recognise that temperature can directly and indirectly impact a community's structure and therefore it's response to environmental change (Petchey *et al.,* 2010). Thus an area of research in need of development is how temperature impacts

species interactions and the mechanisms that mediate these effects through the community. In particular, the strength of species interactions, such as competition and predation, can impact food web stability (Hammill *et al.,* 2015).

The mechanisms that adjust the strength of species interactions under environmental change and consequently food web stability are not fully understood (Hammill *et al.,* 2015). For the progression of robust models of ecological systems, it is imperative to advance our understanding of how indirect responses to temperature are mediated through the food web network and community (Shurin *et al.,* 2012). Petchey *et al*., (2010) conclude that models of temperature effects on community structure and dynamics urgently require considerable development and more empirical data. Furthermore, it is important to assess how trophic structure may alter the effects of temperature and dependent species interactions. Accordingly, the main aim of this chapter is to investigate the direct and indirect impacts of temperature upon community structure and the interaction between these two factors in experimental model systems with a focus on species interactions.

Here I investigate the effects of temperature and community structure on species interactions in experimental aquatic microcosms. Experimental microcosms present the possibility to apply and rigorously test ecological theory and represent a model system (Altermatt *et al.,* 2015). The use of microcosms offers the opportunity to manipulate variables with relative ease and can be highly replicated (Benton *et al.,* 2007). Additionally, the short generation time of the organisms used allows the experiment to reveal long-term trends over many generations. In this study, manipulating the combination of protist competitors and predators in bacteria-protist microcosms enabled the assembly of food webs with varied species interactions and complexity. Bacteria form a significant component of the food web and a key element of microcosms but are often poorly controlled and rarely investigated (but see Jiang and Morin, 2004, Krumins *et al.,* 2006, Beveridge *et al.,* 2010a, 2010b). In some studies, bacteria are ignored as it reasoned that their dynamics are fast in comparison to protists (Fox and Morin, 2001). However, the dynamics of the bacterial community and its response to environmental change could have serious consequences upon the ecological interactions of the microcosm community. Furthermore, bacterial dynamics and functioning are sometimes inferred as the cause of unusual dynamics at higher trophic

levels but are often cited as requiring further study (Jiang and Morin, 2004, Jiang and Krumins, 2006). Ultimately these studies suggest that bacterial population abundances interact with species populations at higher trophic levels. Therefore, I expected that bacterial abundances will be reduced in the presence of bacterivores in comparison to a bacteria-only community. Furthermore, the presence of a top predator would reduce the consumer population; thus bacterial abundances would be greater in the presence of a top predator in comparison to the consumer-bacteria communities.

It has been reported the *Colpidium* displays a negative temperature response, meanwhile *Paramecium* population density is not affected by temperature, despite the positive effect of temperature upon *Paramecium* growth rates (Jiang and Morin, 2004). Therefore, these same trends were anticipated in the protist monocultures. However, this study also examines the interaction of temperature, community structure and community composition. I predicted that community structure and composition would impact species populations through direct and indirect pathways and could alter temperature responses. The *Paramecium-Didinium* community is well documented and known to collapse rapidly (Gause, 1934, Salt, 1974, 1979). Alternatively, the stability of the *Colpidium-Didinium* community was less easily predicted with fewer previous studies. It was expected that the populations would persist longer than those in the *Paramecium-Didinium* community, however it was thought unlikely that the community would persist for the entire experimental period (40 days).

3.2 Methods

Experimental design

Seven communities of varying complexity were established to enable the testing of the relationship between species interactions and temperature (see fig. 3.1). Three distinct trophic levels were represented by bacterial populations, a consumer level of bacterivorous protists and a predator. Species population densities were monitored throughout the food web as the response variable, including the basal bacterial resource. Therefore, the effects of community structure and composition on population density could be assessed through comparing the different communities. Thus, the design of the experiment facilitated the assessment of structural community properties such as number of species, number of trophic levels and species interactions. Furthermore, each community was observed at three temperatures, 22°C, 24°C, and 26°C. The effects of temperature on population abundances could be investigated for each community and the relationship between temperature and community structure evaluated.

The bacterial community was comprised of *S. marcescens subsp. marcescens (*ATCC 13880*), P. fluorescens* SBW25*, and B. cereus* (ATCC 14579), protists used in these experiments include the bacterivores, *Paramecium caudatum* and *Colpidium striatum*, and the predator, *Didinium nasutum.* For each community at each temperature there were 6 replicates, with 126 microcosms in total. Three replicates of the *Paramecium-Colpidium* community were discounted, as during set up for Set A *Paramecium* was mistakenly not added.

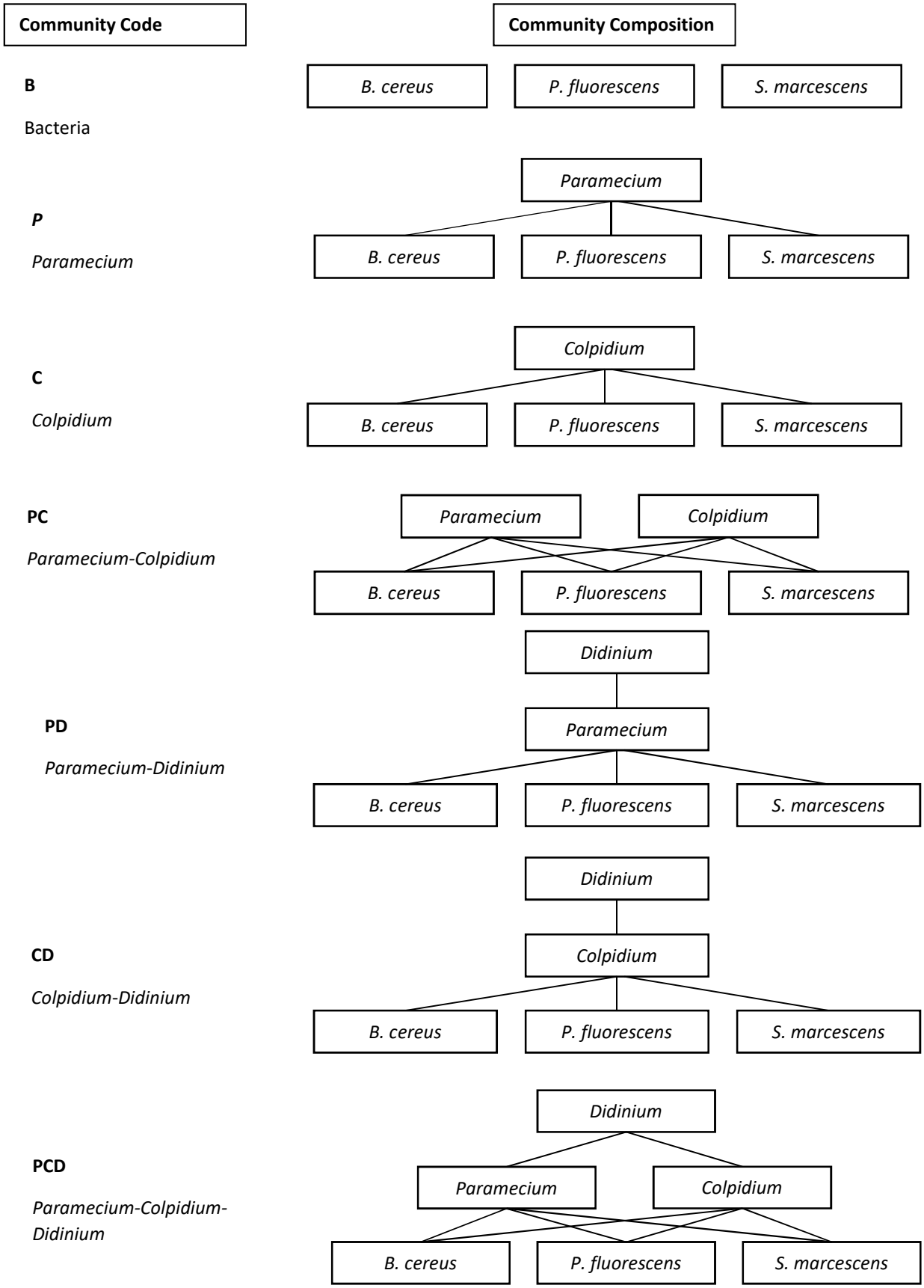


Figure 3.1 Food webs of the seven communities established. Solid lines represent trophic interactions.

Community establishment

Microcosms were 50ml centrifuge tubes containing 25ml of nutrient media. The media was created by adding 0.5g/l of chlorella powder (Naturya Organic Chlorella) to mineral water (Tesco Ashbeck Still Water) and was autoclaved. One sterile barley seed was added to each microcosm to provide a source of long-term nutrients.

Bacteria and protists were maintained in stock cultures at 22°C. Bacteria populations were grown in monocultures at 22°C for 4 days prior to the start of the experiment. qPCR reactions were then used to enumerate the density of each bacterial monoculture. The amount of each bacterial added to each microcosm was modified to give an equal final concentration of each species (30 individuals of each species per 1µl). This ensured an equal starting point for the bacterial communities. Microcosms were randomised before the addition of chlorella media, bacteria media and barley seeds to prevent the occurrence of systematic errors or conditions. Microcosms were observed for 40 days, day 0 was taken as the day these bacterial communities were inoculated.

After the construction of the bacterial community, microcosms were stored at their respective temperatures. Four days later, 100 individuals of the bacterivores, *Paramecium caudatum* and *Colpidium striatum* were added to the respective cultures. These protist stock cultures had been maintained at 22°C with the same bacterial assemblage. The bacterivores were counted using a stereomicroscope to estimate the volume needed for 100 individuals. For *Paramecium* the culture density was 100 individuals per 220µl and for *Colpidium* the culture density was 100 individuals per 8µl.

After four more days, on day 8 (for Set A) and day 9 (for Set B), for the communities with *Didinium,* 10 individuals were counted from the stock culture and added.

To enable the thorough sampling of all species microcosms were split in to two sets, Set A and Set B, with half of the six replicates for each community at each temperature in each set. The sets were staggered by 24 hours in community establishment and sampling.

Community sampling and maintenance

Sampling of bacteria

After day 8 all microcosms were sampled every 2-3 days for the first 18 days, then microcosms were sampled every 3-4 days. The bacterial populations were sampled using qPCR and the protists were counted manually using a stereomicroscope. To collect a sample for qPCR 100µl was removed from each microcosm and was sonicated for 10 minutes. 2µl from each sonicated sample was then removed and added to a qPCR reaction. The qPCR protocol followed the established procedures in Chapter 2. For each microcosm three qPCR reactions were required – one reaction per bacterial species. The qPCR reaction mix consisted of MyTaq^{TM} mix (Bioline), dH_2O, Syto-9 dye, the species-specific primer set and a sample. qPCR reactions were run on each sampling day, 63 reactions per species including a no template control. 2100µl dH_2O was mixed in a 15ml centrifuge tube with 2400µl MyTaq mix and 192µl dye. This was then split into three aliquots of 1530µl, and labelled as either, BC, PF or SM. 64µl of a primer pair was added to the respective aliquot. The qPCR reaction mix was then added to a qPCR tray in 23µl aliquots. A 2µl sample could then be added to the relevant well. This resulted in each reaction made up to 25µl, constructed with 12.5µl MyTaq^{TM} mix, 1µl dye, 1µl of primers and 2µl of sample.

B. cereus was not detected consistently throughout the experiment. The level of detection was limited to roughly 50 individuals per 1µl. As discussed in Chapter 2, detection becomes limited at low population levels, either due to *B. cereus* characteristically forming chains when multiplying or as a result of bacteria collecting around particulate matter, especially with the formation of biofilms. It is assumed that where *B. cereus* was not detected it was therefore at a population density less than 50 individuals per 1µl.

Sampling of protists

To estimate the abundance of each protist species, microcosms were gently swirled, a known volume was removed with the use of a Gilson pipette and individuals were counted under a microscope. A portion (2.5ml, 10%) of the media was removed and replaced with sterile chlorella media for the first time on day 18 and then weekly thereafter. The removal and replacement of media maintained the level of nutrients

available and prevented the build-up of metabolic waste (Worsfold *et al.,* 2009). The media was not replaced for the first 18 days to avoid the interference with protist dynamics, particularly *Didinium*, at low densities. Evaporative water loss was replaced at the same time to ensure microcosms were consistently 25ml.

Data and analysis

Peak abundance, representing carrying capacity, was analysed in response to temperature and community composition. Two-way ANOVA was used to determine the effects of temperature, community and the interaction between these two factors. All analyses were carried out using R version 3.3.0. (R Core Team, 2016).

In order to enumerate the bacteria, the noise and threshold lines were set to 1000 and 10,000 for all data. The qPCR software identified a cycle as a fail when no fluorescence was detected. A fail can be the result of no sample DNA being present in the reaction or it can be a failure of the reaction due to any of its components. Therefore, a 'FAIL' result was treated as no sample was measured, rather than the absence of the species in the microcosm. Additionally, to interpret the qPCR data where a product was formed, data were rejected if the cycle had not formed a correct, species-specific product or a non-specific product. This was done by considering the dissociation curve analysis. Each primer product produced a steep drop at a known temperature (cycle number) shown in figure 2.6. The reaction was treated as an NA if the dissociation curve analysis showed a gradual decline in fluorescence or a steep drop in fluorescence 5 cycles either side of the known cycle number.

3.3 Results

3.3.1 Effects on *Colpidium*

The population density of *Colpidium* through time for each community at each temperature is shown in figure 3.2. Temperature and community composition interacted to affect the peak population of *Colpidium* (two-way ANOVA, significant interaction $F_{3, 61} = 4.59$, $P = 0.00584$). All populations of *Colpidium* initially increased rapidly. Considering the *Colpidium*-only community (C, fig. 3.2) it can be seen that there was a negative effect of increasing temperature upon peak abundance (fig. 3.3). However, the initial rate of growth is extremely similar across all three temperatures *at* ~3,700 per 1ml per day. The maximum population density of *Colpidium* when alone, on average, reached approximately 40,000 per 1ml at 22°C, 30,000 per 1ml at 24°C and 20,000 per 1ml at 26°C. In the *Paramecium*-*Colpidium* community (PC) the *Colpidium* population was lower than when alone. This effect was also dependent upon temperature; *Colpidium* grew to an average population density of approximately 30,000 individuals per 1ml at 22°C, 20,000 individuals at 24°C and 15,000 individuals at 26°C (fig. 3.2). *Colpidium* was always far more abundant than *Paramecium* when cultured together, regardless of temperature (fig. 3.2, 3.4). In the *Colpidium*-*Didinium* community (CD), predation by *Didinium* reduced the *Colpidium* population density, relative to the *Colpidium*-only community (fig. 3.3). This effect was also temperature-dependent, where the *Colpidium* population was reduced more at 22°C, so the population of *Colpidium* reached a higher peak abundance at 24°C (approximately 32,000 per 1ml), then 22°C (approximately 25,000 per 1ml) and then 26°C (approximately 21,000 per 1ml). However, the negative effect of *Didinium* on *Colpidium* was not as great as the negative effect of *Paramecium* – the population of *Colpidium* was, on average, always higher in the CD communities than the PC communities. In the *Paramecium*-*Colpidium*-*Didinium* community (PCD), *Colpidium* populations initially increased to a mean population of ~15,000 per 1ml (fig. 3.2). The effect of having both *Didinium* and *Paramecium* present was no more negative than only having *Paramecium* present – in some cases, *Colpidium* populations were higher than with *Paramecium* alone (this was presumably due to the negative effect that *Didinium* predation had on both prey species).

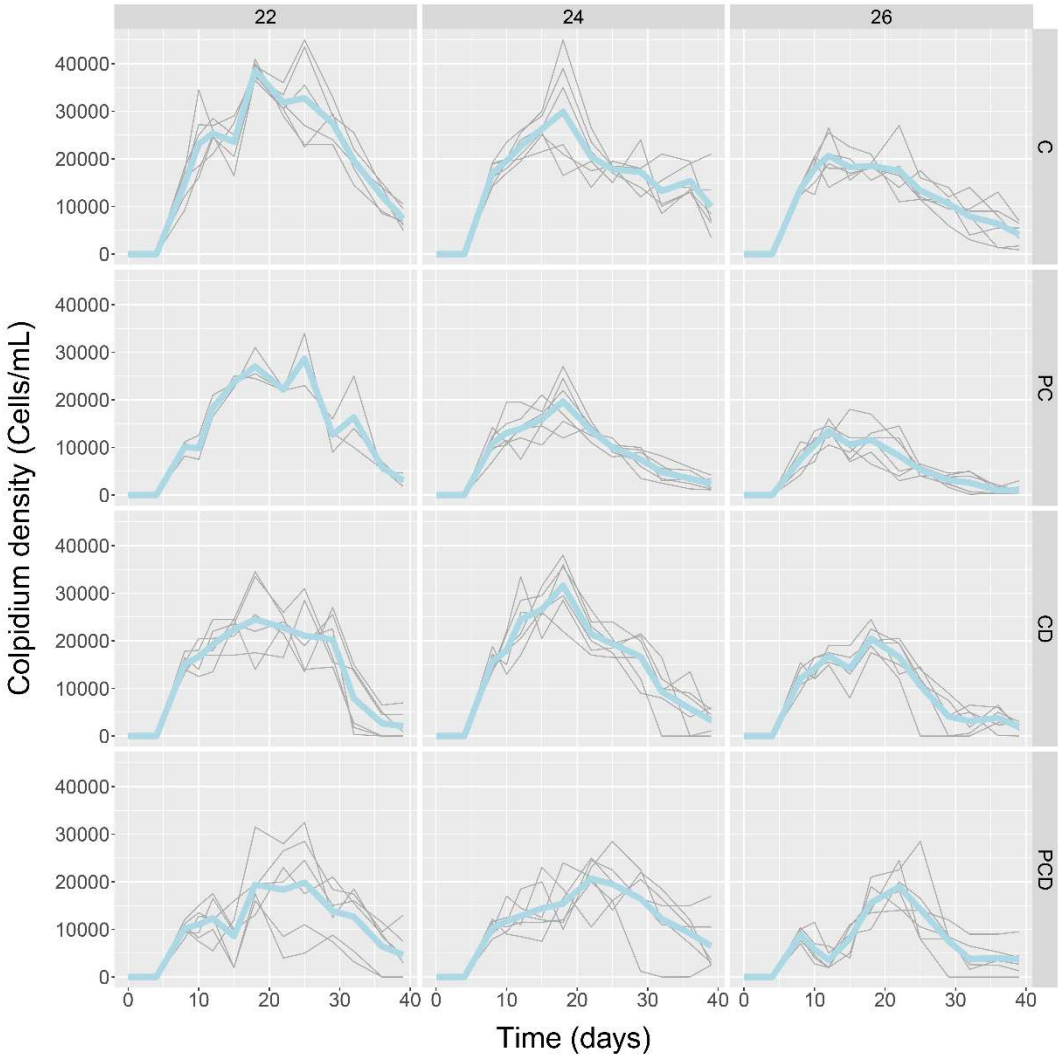


Figure 3.2: The population density for *Colpidium* shown in each community at each temperature treatment. Light grey population densities show results per replicate, the thick light blue population density is the mean population density.

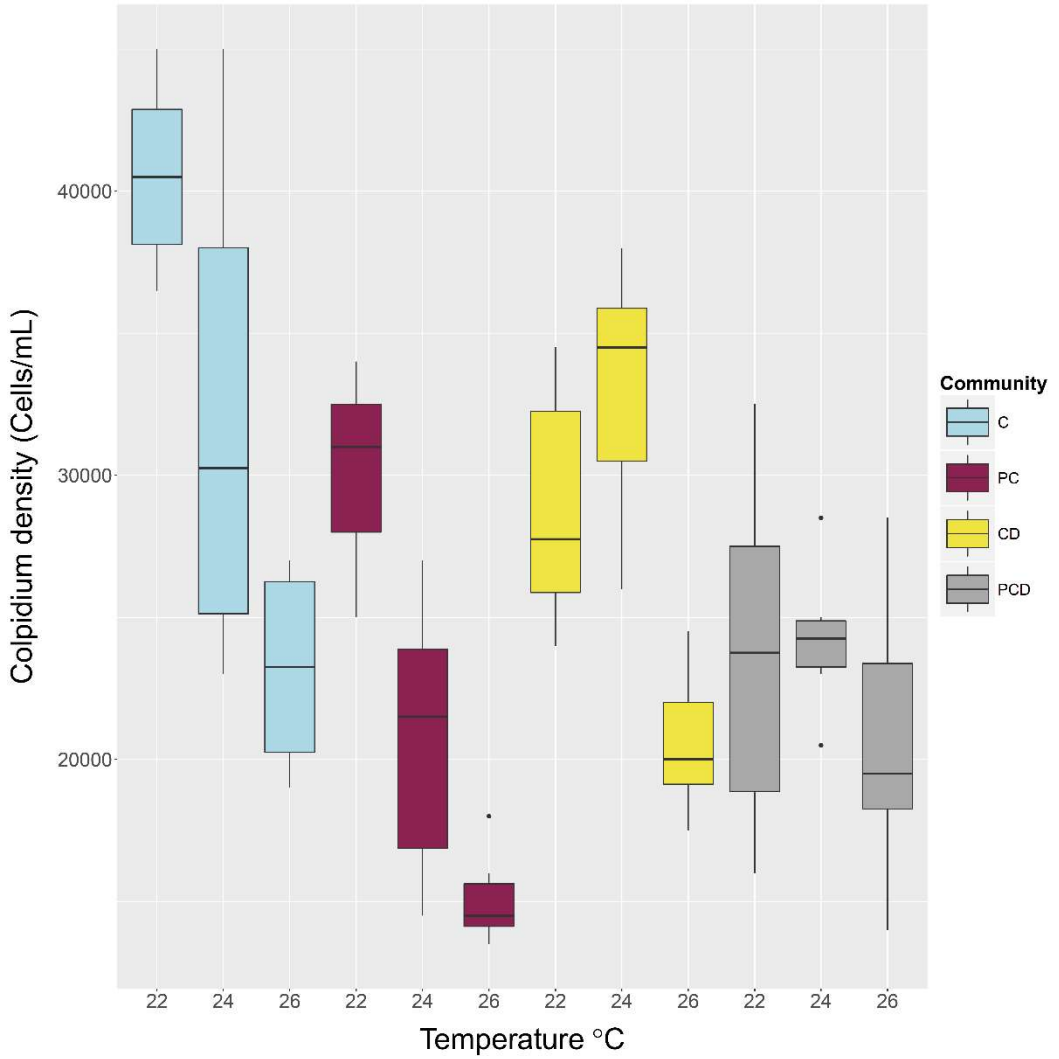


Figure 3.3: The effects of temperature and community on the maximum population density of *Colpidium*. Box plots show a five number summary of peak abundance.

3.3.2 Effects on *Paramecium*

The population density of *Paramecium* through time for each community composition at each temperature is shown in figure 3.4. The effects of temperature and community structure upon *Paramecium* are shown in figure 3.5. *Paramecium* was approximately an order of magnitude less abundant than *Colpidium*. There was no significant main effect of temperature on the *Paramecium* population (two-way ANOVA, temperature: $F_{1, 61}$ = 3.24, P = 0.0770, see P community, fig. 3.5). *Paramecium* grew slightly faster and to a higher abundance at 24°C and 26°C than at 22°C (fig. 3.4). Between day 4 and 8, the rate of growth at 24°C and 26°C was ~170ml/day, whilst at 22°C was ~100ml/day. In the PC community (fig. 3.4). The mean density of *Paramecium* was significantly reduced in the presence of *Colpidium* (fig. 3.5). However, there was no significant interaction between temperature and community ($F_{3, 61}$ = 0.755, P= 0.524). *Didinium* had a very dramatic effect on the population of *Paramecium*. In the PD community, the addition of *Didinium* after four days of *Paramecium* growth led to the population being consumed within a few days (fig. 3.4). At 22°C and 24°C, *Paramecium* went extinct within 4 days. At 26°C, *Paramecium* went extinct within 2 days. Where *Colpidium* was also present (PCD community) the effect on *Paramecium* was even more negative – *Paramecium* grew very slowly in the four days before *Didinium* was added and all individuals were consumed within 2 days after the addition of *Didinium*. Unsurprisingly, with such dramatic effects of predation, *Paramecium* abundance was significantly affected by community composition; $F_{3, 61}$ = 175.3, P <0.0001.

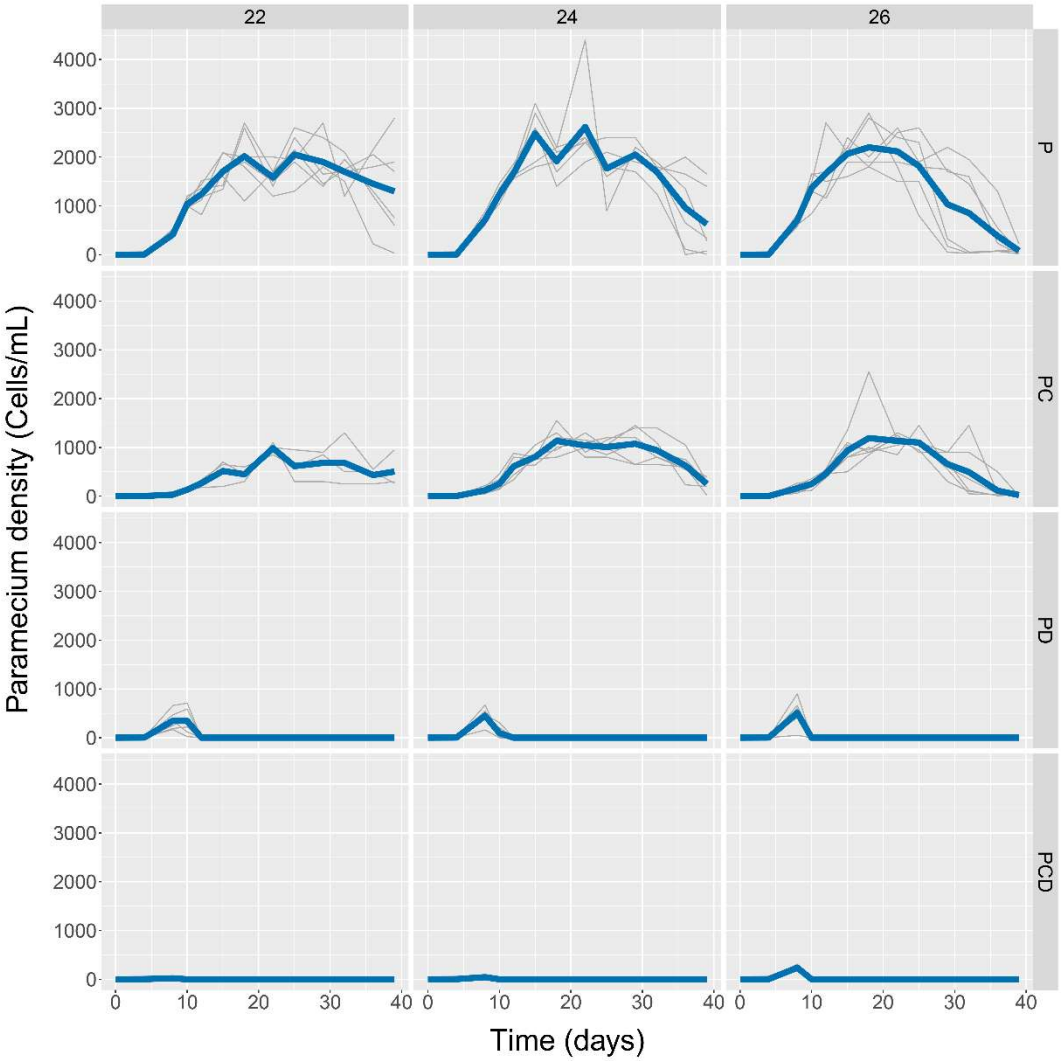


Figure 3.4: The population density for *Paramecium* shown in each community at each temperature treatment. Light grey population densities show results per replicate, the thick blue population density is the mean population density.

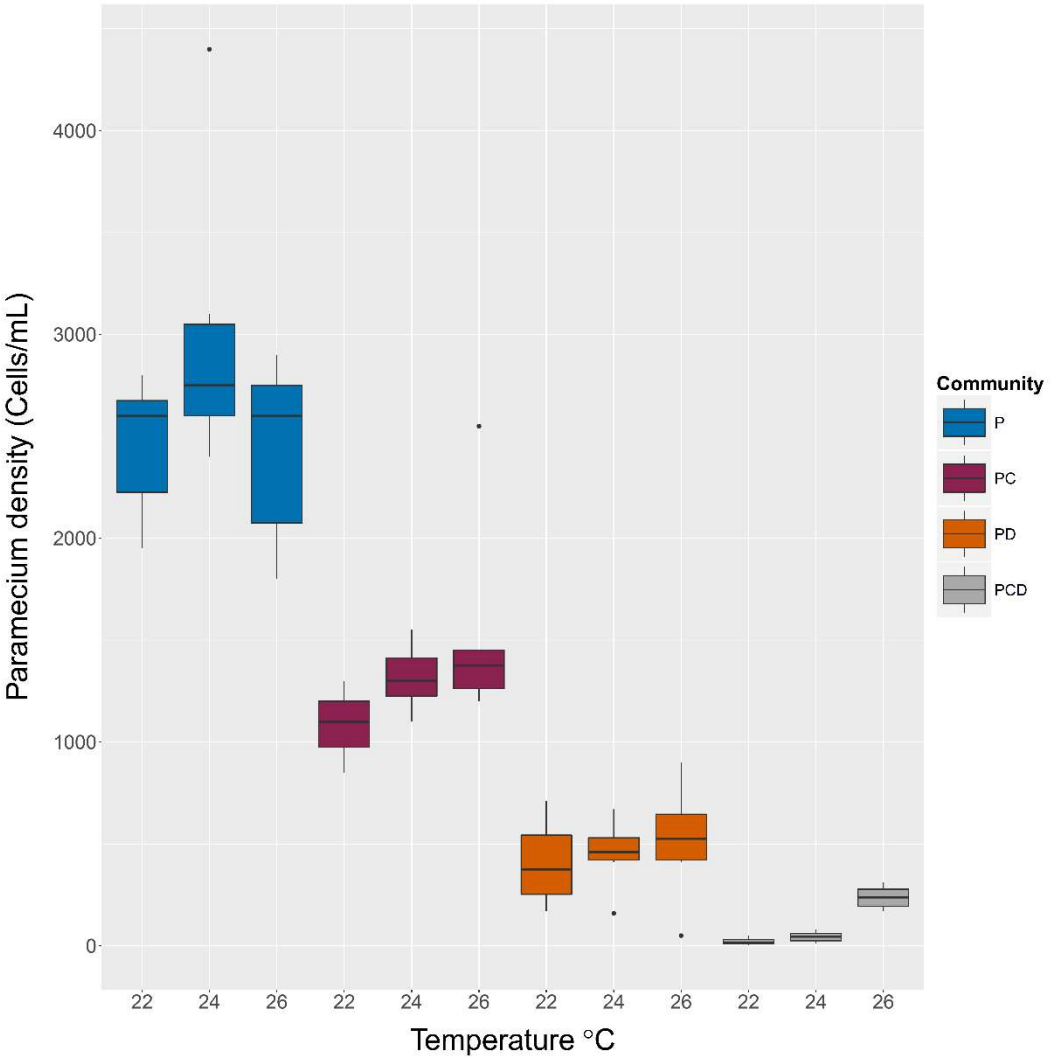


Figure 3.5: The effects of temperature and community on the maximum population density of *Paramecium*. Box plots show a five number summary of peak abundance.

3.3.3 Effects on *Didinium*

The population density of *Didinium* through time for each community at each temperature is shown in figure 3.6. The effects of temperature and community structure upon *Didinium* are shown in figure 3.7. The community had a significant effect upon the *Didinium* population (two-way ANOVA: $F_{2, 48} = 55.8$, $P < 0.0003$, fig 3.7). *Didinium* populations reached much higher density with *Paramecium* than with *Colpidium* (fig. 3.7). *Didinium* temperature response was community dependent (fig.3.7), displaying a slight negative temperature response with *Colpidium* and a positive temperature response with both prey species present. With *Colpidium* present, (CD community) *Didinium* mean population initially increased to 14 per 1ml at 22°C, and 3 per ml at 24°C and 26°C. At 22°C the mean population of *Didinium* remained at ~10 individuals per 1ml from day 15 – 25 and reached a maximum population density of 35 per 1ml on day 32. At 24°C and 26°C *Didinium* populations remained between 5 – 10 per 1ml. Although the population density at 24°C did increase to 21 individuals per 1ml on day 36. At 22°C *Didinium* remained present until the end of the experiment. At 24°C *Didinium* went extinct in one out of 6 replicate on day 25. At 26°C, *Didinium* went extinct in 2 out of 6 replicates on day 25, *Didinium* went extinct on day 29 in one replicate and extinct in a further replicate on day 36. This means that by day 39, no extinctions of *Didinium* were recorded at 22°C, one extinction was recorded at 24°C and four extinctions were recorded at 26°C. In the *Paramecium-Didinium* community, all *Didinium* populations increased initially to a population of ~200 per 1ml (fig. 3.6). This was faster at 26°C, (115 per ml/day) than at 24°C and 22°C, (92 and 42 per ml/day respectively). Following the extinction of *Paramecium,* at 22°C, *Didinium* went extinct on day 18 in 4 out of 6 replicates and on day 22 in 2 out of 6. At 24°C and 26°C *Didinium* went extinct on day 18 in all replicates. In the *Paramecium-Colpidium-Didinium* community on day 10, *Didinium* populations increased to a mean population of 36 per ml at 22°C, 64 at 24°C and 177 at 26°C. In all replicates the *Didinium* population declined from day 10 to day 12; the size of the population peak on day 10 was amplified with increasing temperature. At 22°C the mean population peaked again on day 18 at 39 per ml and on day 32 at 27 per ml, although there was slight variability between replicates. At 24°C the mean population density peaked on day 29 at 28 per ml, replicates showed variability in the magnitude of this peak.

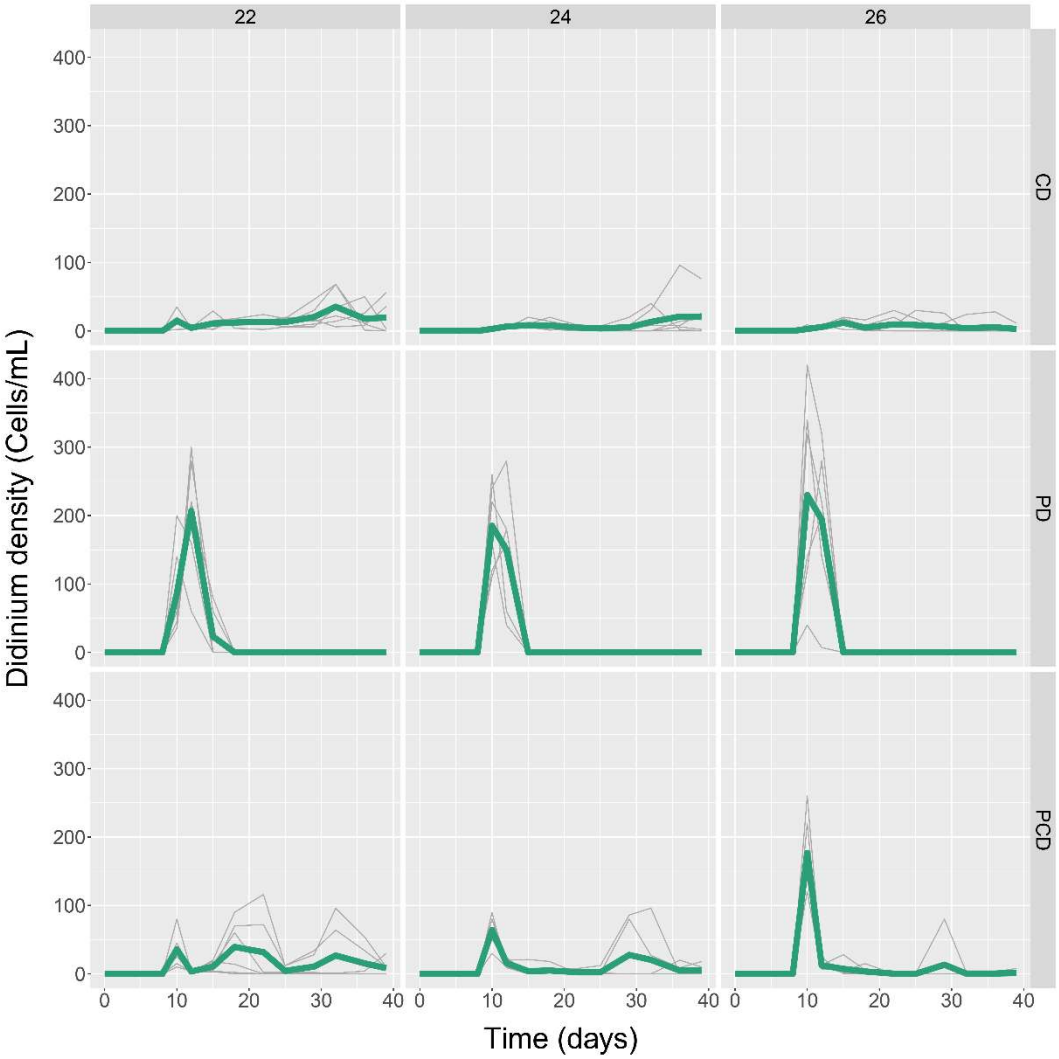


Figure 3.6: The population density for *Didinium* shown in each community at each temperature treatment. Light grey population densities show results per replicate, the thick green population density is the mean population density.

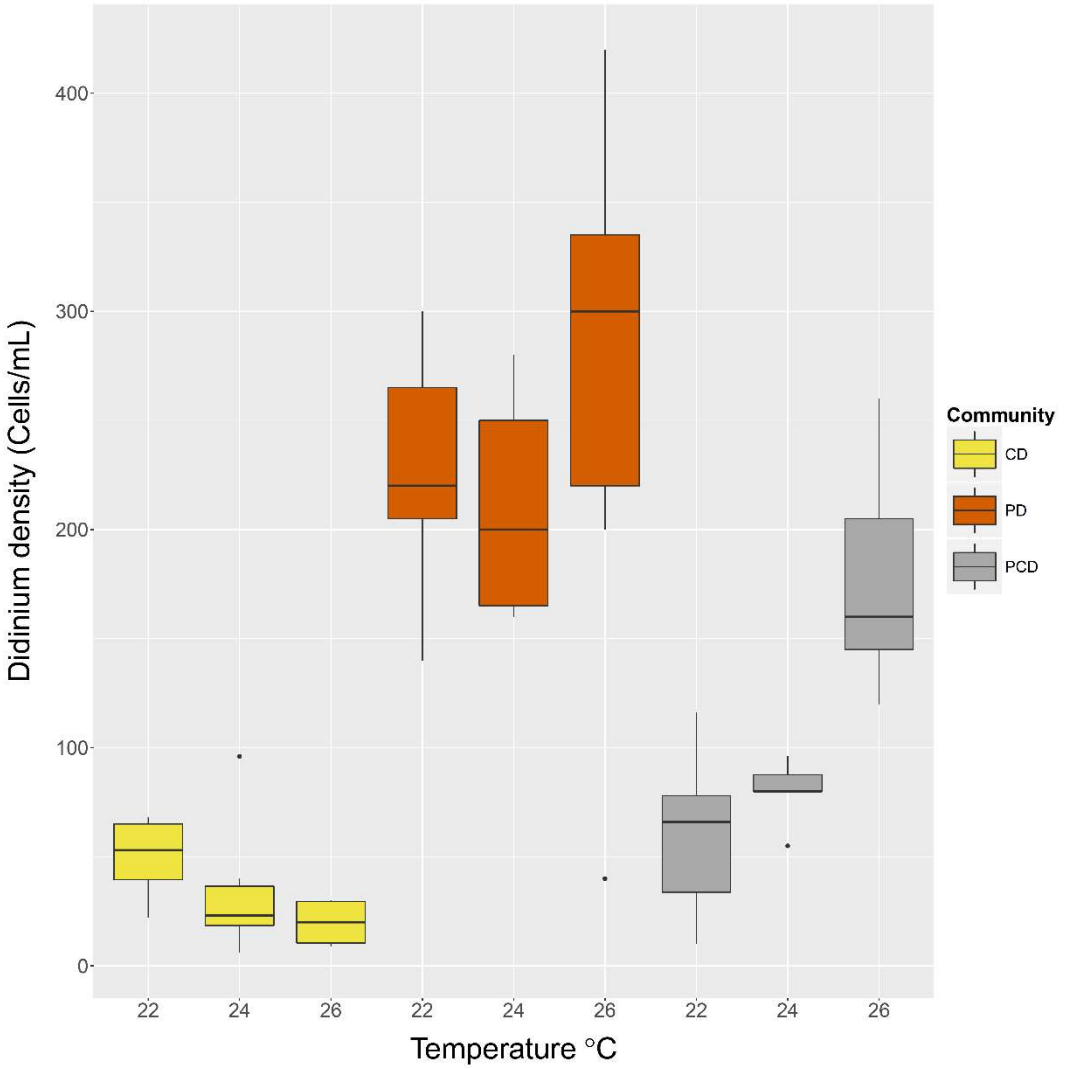


Figure 3.7: The effects of temperature and community on the maximum population density of *Didinium*. Box plots show a five number summary of peak abundance.

3.3.4 Effects on the bacterial community

B. cereus had a density of less than 50 individuals per µl for the duration of the experiment at all temperatures when detected and was sometimes undetectable (data not shown). Due to the low density and thus low detectability of *B. cereus* the effects of community structure upon this bacterial species cannot be judged.

In the bacteria-only community (fig. 3.8) the mean population density of *P. fluorescens* peaked at 121,137 per µl on day 18 at 22°C, 316,166 on day 39 at 24°C and 85,200 on day 32 at 26°C. Meanwhile the population density of *S. marcescens* peaked at 13,031 per µl on day 32 at 22°C, 86,965 on day 32 at 24°C and 381,374 on day 39 at 26°C. At 22°C and 24°C, *P. fluorescens* was the most abundant, then *S. marcescens*, and the least abundant was *B. cereus*. This was also the order of abundance for the first 18 days at 26°C, but on day 22 *S. marcescens* became more abundant than *P. fluorescens* and then remained the most abundant bacteria species for the majority of the experiment thereafter. The population densities remained relatively stable and did not exhibit any signs of depleted energy availability by the end of the experiment. From day 12 at 24°C and 26°C, the population of *S. marcescens* increased, from approximately 600 per µl, peaking at approximately 30,000 per µl on day 32 and 29 respectively.

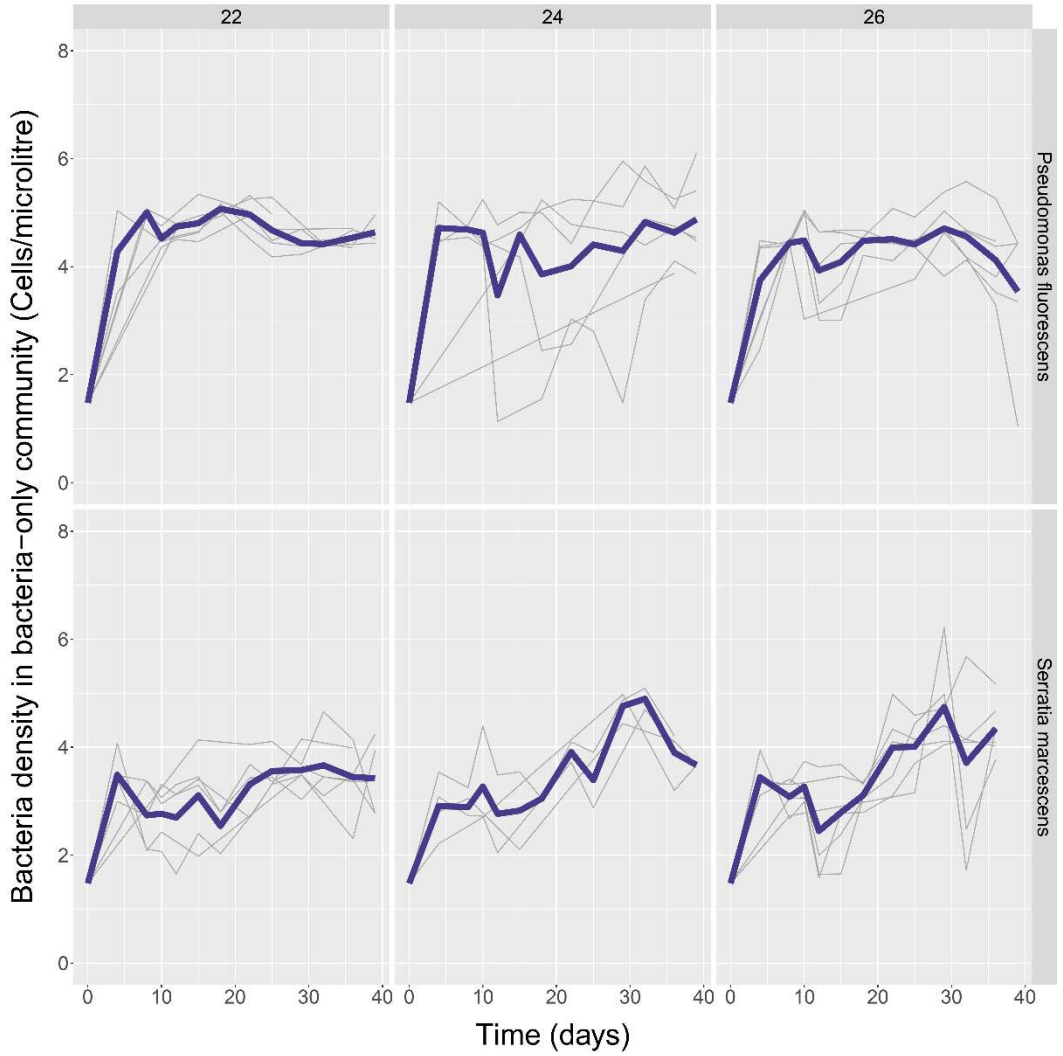


Figure 3.8. Population density (Log_{10} per μl) over time for, *P. fluorescens fluorescens* and *S. marcescens* in the Bacteria-only community at the three temperature treatments. Grey population densities show the population for each replicate, thick purple shows the mean population density.

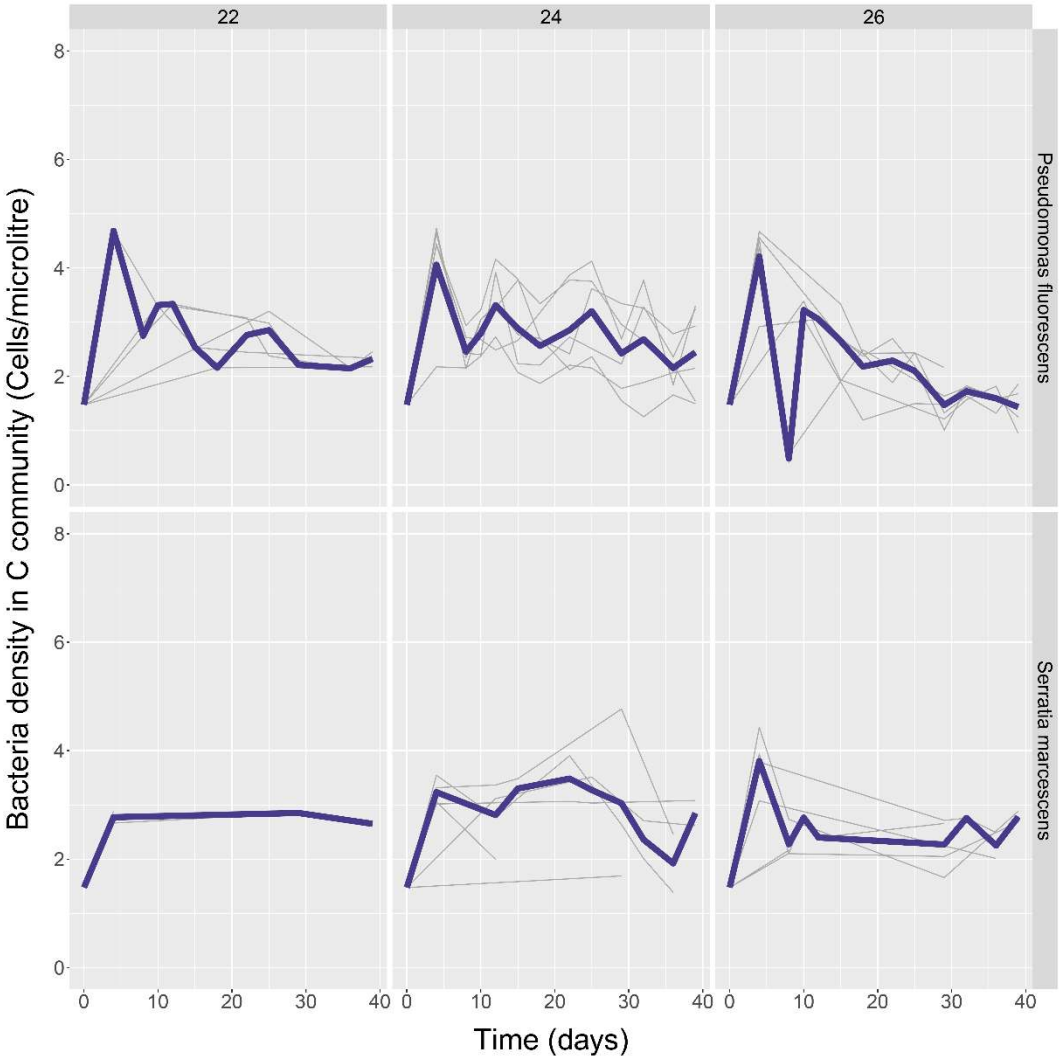


Figure 3.9. Population density (Log_{10} per μl) over time for *P. fluorescens* and *S. marcescens* in the *Colpidium*-only community at the three temperature treatments. Grey population densities show the population for each replicate, thick purple shows the mean population density.

After the addition of *Colpidium*, bacterial populations began to decline (fig. 3.9). In comparison to figure 3.8, figure 3.9 shows the presence of *Colpidium* reduced the abundance of the bacterial species, *P. fluorescens* and *S. marcescens*. Where *Colpidium* was absent, *P. fluorescens* was the most abundant bacteria at 22°C and 24°C, and for the first 22 days at 26°C before *S. marcescens* became dominant (fig 3.8). Where *Colpidium* was present *S. marcescens* and *P. fluorescens* remained equally abundant. The population density of *P. fluorescens* generally declined over time, whilst the population density of *S. marcescens* appeared to remain fairly static.

Figure 3.10 shows the population density of *P. fluorescens* and *S. marcescens* in the *Paramecium* community. The mean population density of *P. fluorescens* tended to decline over time, although initially reached a higher density at 24°C and 26°C. It is noteworthy that some replicates at 22°C produced similar peaks. The population density of *S. marcescens* fluctuated between 200 and 10,000 per µl at all temperatures. In comparison to figure 3.8 (Bacteria-only), figure 3.10, shows that the presence of *Paramecium* reduced the population density of *P. fluorescens* and *S. marcescens.* In comparison to figure 3.9 (*Colpidium* community) it appears that *Paramecium* and *Colpidium* reduced the bacteria populations by a similar factor.

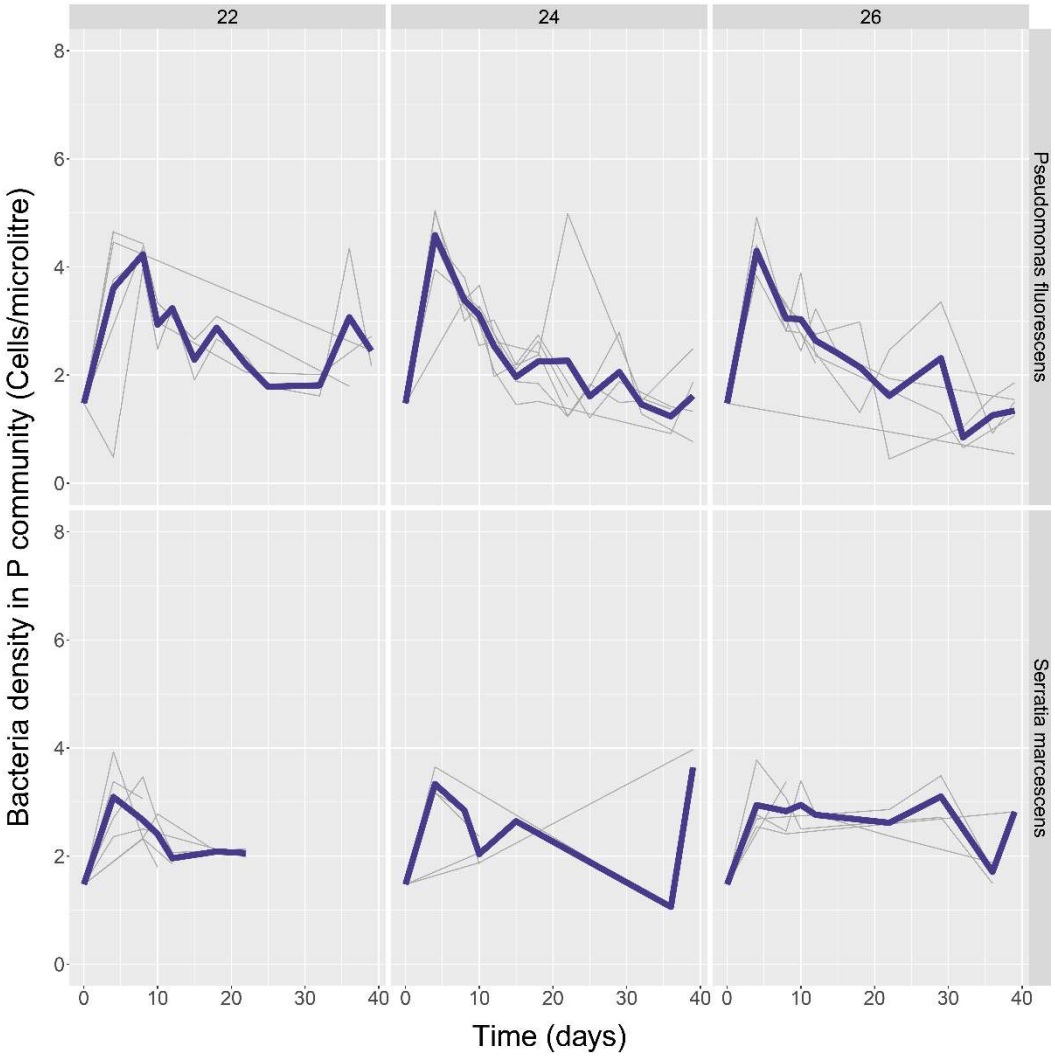


Figure 3.10. Population density (Log_{10} per μl) over time for *P. fluorescens* and *S. marcescens* in the *Paramecium*-only community at the three temperature treatments. Grey population densities show the population for each replicate, thick purple shows the mean population density.

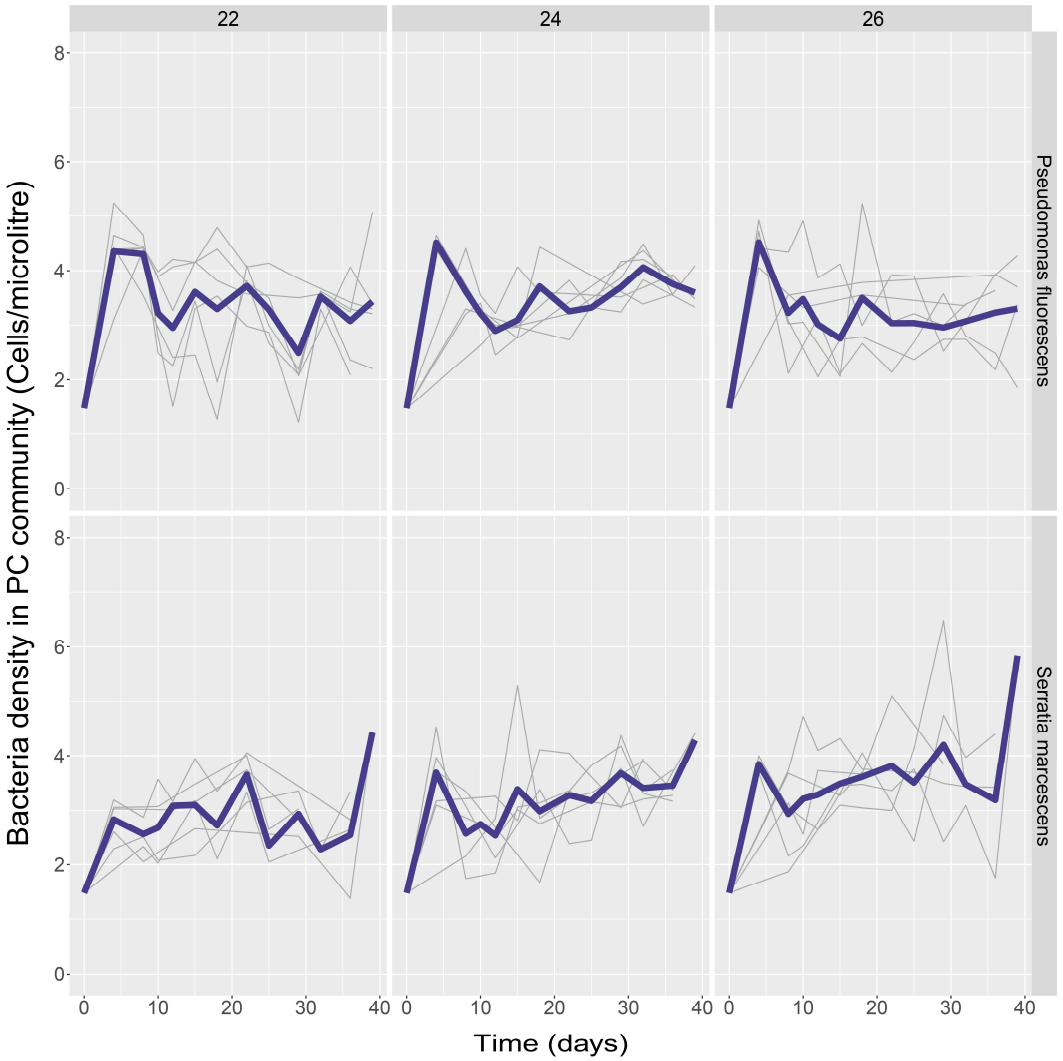


Figure 3.11. Population density (Log_{10} per µl) over time for *P. fluorescens* and *S. marcescens* in the *Paramecium-Colpidium* community at the three temperature treatments. Grey population densities show the population for each replicate, thick purple shows the mean population density.

Bacterial data for the *Paramecium-Colpidium* community are shown in figure 3.11. After the addition of the protists the population of *P. fluorescens* decreases and fluctuates between 100 to 1000 per µl. Meanwhile the mean population of *S. marcescens* gradually increases after the addition of protists, although there is large variation between replicates.

Bacterial data for the *Colpidium-Didinium* community are shown in figure 3.12. The population densities of both bacteria declined over time. *P. fluorescens* declined to lower densities at higher temperatures, whilst *S. marcescens* declined to a similar density at all temperatures. In comparison to figure 3.9 (*Colpidium* community), figure 3.12, shows that the presence of *Didinium* reduced the negative impact of *Colpidium* on the population density of *P. fluorescens* and *S. marcescens.*

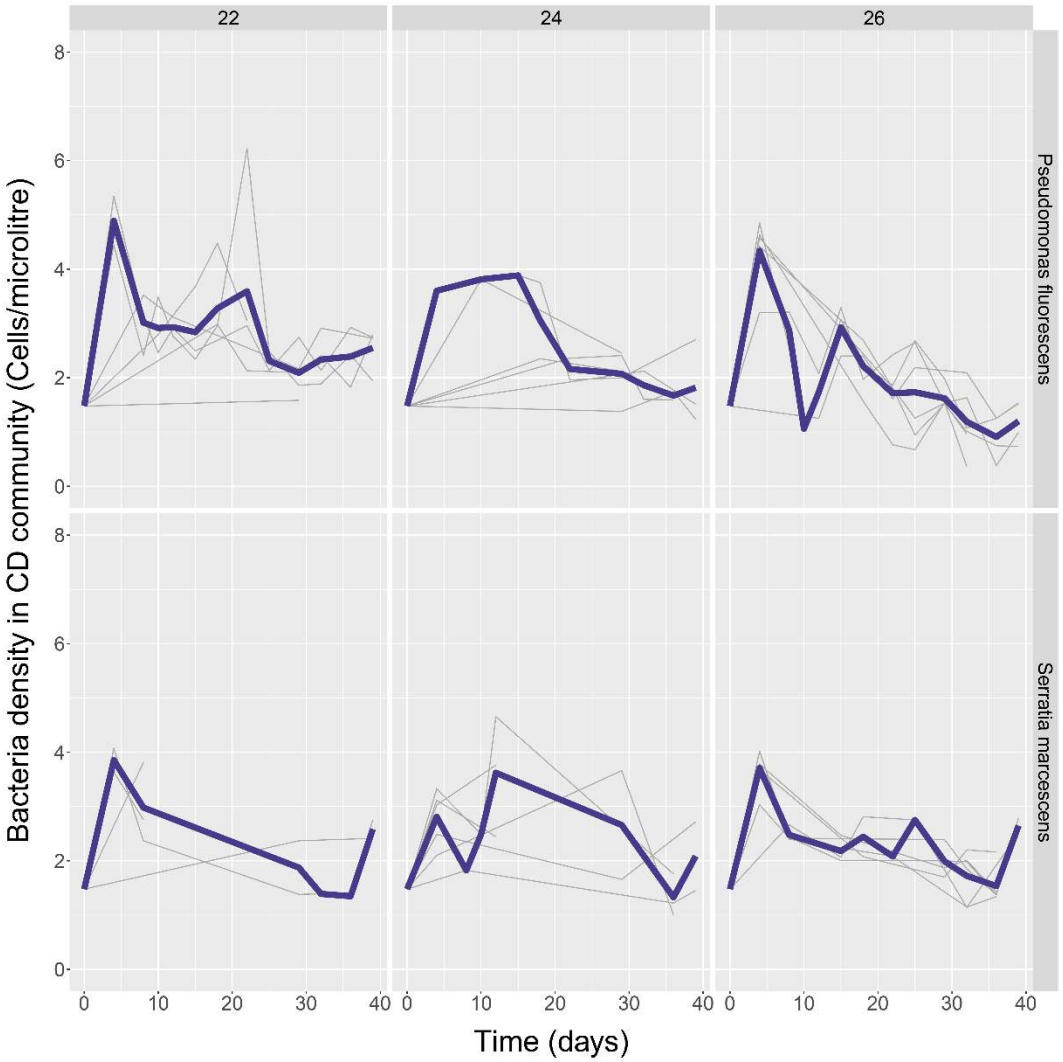


Figure 3.12. Population density (Log_{10} per μl) over time for *P. fluorescens* and *S. marcescens* in the *Colpidium-Didinium* community at the three temperature treatments. Grey population densities show the population for each replicate, thick black shows the mean population density.

Bacterial data for the *Parameciu*m-*Didinium* are shown in figure 3.13. *P. fluorescens* and *S. marcescens* rapidly increase their population density on day 4. However, the population abundance then decreased due the addition of *Paramecium* on day 4. In comparison to figure 3.10 (Paramecium), figure 3.13, shows that the addition of *Didinium* on day 8 reduced the negative impact of *Paramecium* on the population density of *P. fluorescens* and *S. marcescens.* The community collapsed to Bacteria-only by day 22 (figs. 3.4, 3.6). However, in comparison to figure 3.8 it can be seen that despite the biological removal of higher trophic levels, the bacteria populations did not recover to a similar density as in the bacteria-only community. *P. fluorescens* showed little sign of recovery. The population density of *S. marcescens* appeared to increase gradually with time. It is noteworthy that despite short generation times both species of bacteria did not rapidly recover from the biological disturbance.

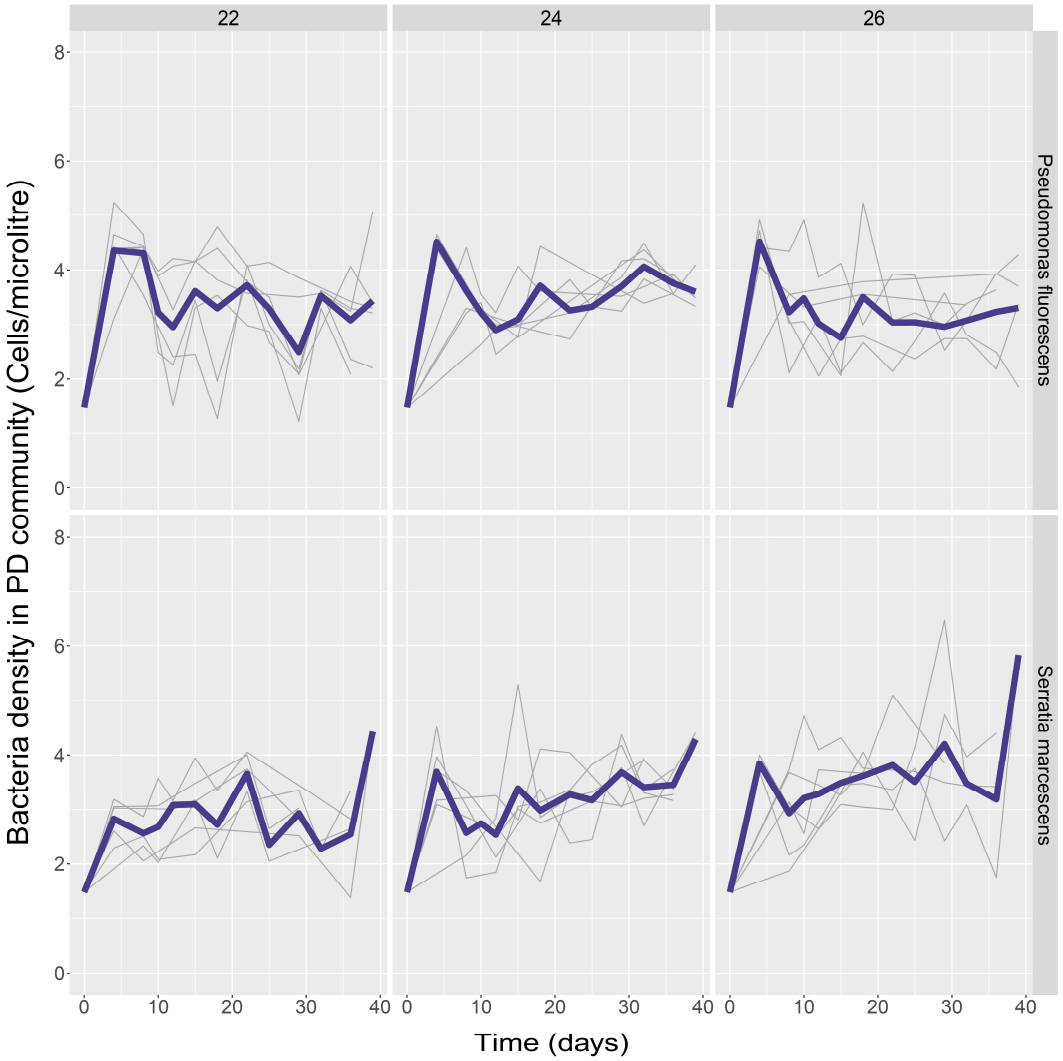


Figure 3.13. Population density (Log_{10} per µl) over time for *P. fluorescens* and *S. marcescens* in the *Paramecium-Didinium* community at the three temperature treatments. Grey population densities show the population for each replicate, thick purple shows the mean population density.

Bacterial data for the *Paramecium-Colpidium-Didinium* are shown in figure 3.14. The mean population density of *P. fluorescens* declined over time at 24°C and 26°C. This trend is not as apparent at 22°C, however there is a high amount of variation between replicates. The mean population density of *S. marcescens* fluctuated to 10,000per µl. There are fewer data points for *S. marcescens* in this community due to samples being classified as fails or 'NA'.

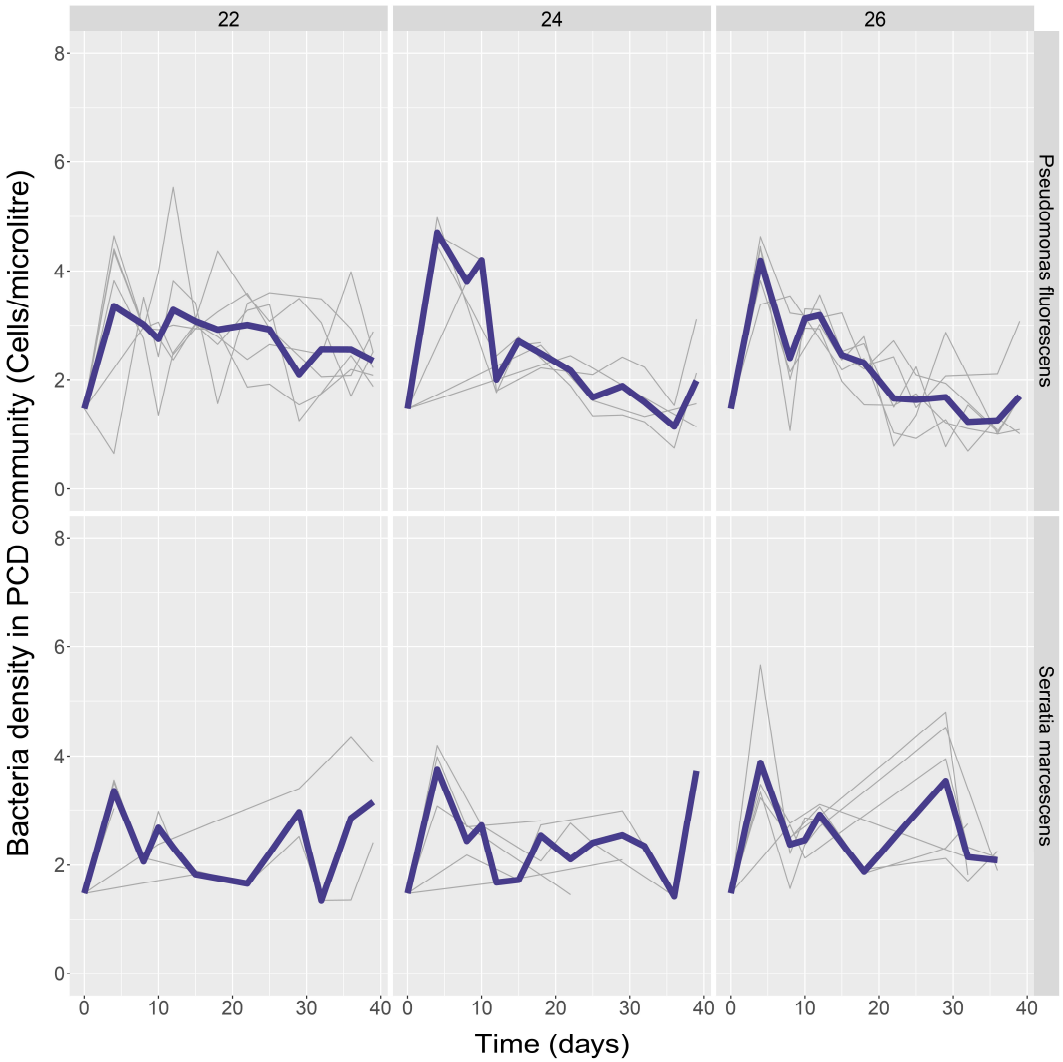


Figure 3.14. Population density (Log_{10} per µl) over time for *P. fluorescens* and *S. marcescens* in the *Paramecium-Colpidium-Didinium* community at the three temperature treatments. Grey population densities show the population for each replicate, thick black shows the mean population density.

3.4 Discussion

My results show that community response is significantly impacted by temperature, and the community structure. In these communities the effects of community composition and temperature was strongly mediated via indirect interactions. These results therefore support the findings of Jiang and Morin (2004) and Beveridge *et al.,* (2010a), which highlight the importance of indirect interactions when considering community response to environmental change. The statistical interaction of community composition and temperature determined population density and persistence. Therefore, in order to understand community response to climatic change, it is critical to consider species-specific response to both temperature and community structure and the interaction of these factors, plus the implications for the community via direct and indirect pathways.

3.4.1 Higher trophic levels

Temperature and community structure affected the population density and persistence of the protists and ultimately community stability. Generally, *Colpidium* was roughly ten-fold denser than *Paramecium*, and *Paramecium* was roughly ten-fold denser than *Didinium.* This study found a significant negative temperature effect upon *Colpidium,* where increasing temperature caused a decrease in population abundance and no effect of temperature upon the population abundance of *Paramecium.* This study also found that when in competition, *Paramecium* and *Colpidium* were both negatively impacted and the competitive interaction indirectly interacted with temperature to alter the response of *Paramecium.* The same temperature responses for the monocultures are reported in Jiang and Morin (2004), Jiang and Kulczyki (2004) and Leary and Petchey (2009). It is also reported in Jiang and Morin (2004) and Jiang and Kulczyki (2004) that competition with *Colpidium* alters the response of *Paramecium* to temperature.

Predator-prey dynamics and temperature response

In these communities, there is evidence for strong interactions between trophic levels determining predator-prey response and mediating the effects of temperature. For example, in the *Colpidium-Didinium* community, *Didinium* reached greater population densities at the lower temperature (fig 3.6). It therefore appeared that the population density of *Didinium* increased with decreasing temperature. Meanwhile in the *Paramecium-Didinium* community, *Didinium* reached a higher population density with increasing temperature (fig 3.6). These contrasting dynamics of *Didinium* are likely an indirect response mediated by the prey species. In the *Colpidium-Didinium* community, the temperature response of *Colpidium* caused there to be greater prey availability for *Didinium* at cooler temperatures. Yet, in the *Paramecium-Didinium* community there was greater prey availability at higher temperatures on day 8. This suggests that *Didinium* population density was controlled more strongly by prey availability than the direct effects of temperature. In the *Paramecium-Colpidium-Didinium* community, the indirect effect from competition caused by *Colpidium* on *Paramecium* altered the temperature response of *Paramecium.* Where *Colpidium* was less dense at higher temperatures, there was less of a negative competitive effect on *Paramecium.* This indirect effect caused a boost in the *Didinium* population at 26°C where there was a denser initial population of *Paramecium.*

Additionally, considering the *Paramecium-Didinium* community, the results suggest that a small change in predator density had a magnified effect upon the prey population. On day 10, the *Didinium* population density was highest at 26°C, with a population density of 230 individuals per 1ml. This dense population caused the complete extinction of *Paramecium* by day 10. In comparison the population density of *Didinium* at 24°C was slightly smaller, with 185 individuals per 1ml, and as a result there were still *Paramecium* present on day 10 with an average population density of 97 individuals per 1ml. On the same day the population density of *Didinium* was much lower at 22°C, with 86 individuals per 1ml. As a result of the reduced effect of predation the average population density of *Paramecium* was comparatively very high, at 344 individuals per 1ml. This high population density of *Paramecium* at 22°C on day 10, could explain why the *Didinium* population density was able to continue to increase on day 12 at 22, while at 24°C and 26°C the population declined.

Prey species-specific traits determine coexistence and stability

Contrasting the *Paramecium-Didinium* and *Colpidium*-Didinium communities revealed the importance of species-specific traits. These communities had the same network structure, but resulted in very different community dynamics. The predator-prey relationships could have been highly dependent upon species-specific traits, such as body size, swimming speed and rate of growth. The *Didinium-Paramecium* community was highly unstable, as *Didinium* rapidly exhausted the *Paramecium* prey, causing both their extinction. This may be unsurprising due to the amount of studies demonstrating the instability of this predator-prey system (Gause, 1934, Gause *et al*., 1936, Luckinbill, 1973, Salt, 1974, 1979). Meanwhile, the *Colpidium-Didinium* community demonstrated remarkably stable dynamics in terms of persistence and the variability of population densities. This leads to the question; what are the differential factors between *Paramecium* and *Colpidium* which altered the ability of the system to coexist?

There is a trade-off for both species between abundance and body size. *Colpidium* is far denser than *Paramecium,* however the body size of individual *Colpidium* is much smaller. Due to the small size of *Colpidium* it is likely that *Didinium* would need to capture more *Colpidium* than *Paramecium* to obtain the same amount of energy. When initially added to the microcosm it is therefore possible that *Didinium* could obtain energy faster where there was dense *Paramecium* rather than dense *Colpidium.* These results may provide evidence for this at higher temperatures as on day 10 the population density of *Didinium* was roughly 100 times greater in the presence of *Paramecium* compared to *Colpidium* (fig. 3.6, 3.7). It should be noted that at 22°C, this difference in the *Didinium* population density was much smaller, although the *Didinium* population was still 6 times greater in the presence of *Paramecium* than *Colpidium*. This was likely due to the different temperature responses of the prey species, as *Paramecium* was less abundant at 22°C. Furthermore, when prey density is low, the body size and swimming speed of an individual could determine its chances to evade predation, and the rate of growth determines the species ability to rebound from a near extinction event. *Paramecium* has a comparatively large body size, slow swimming speed and slow rate of growth. The combination of these factors could therefore mean that when at low density the *Paramecium* population had a very poor chance to recover and was therefore rapidly exhausted. On the other hand, there could have been

'strength in numbers' for *Colpidium*. On day 8 the population density of *Colpidium* in comparison to *Paramecium* was roughly 43 times greater at 22°C, 35 times greater at 24°C and 24 times greater at 26°C.

In comparison to the *Paramecium-Didinium* cultures, the *Paramecium* extinctions in the *Paramecium-Colpidium-Didinium* community, were recorded earlier in the presence of *Colpidium* at all temperatures. This was likely due to the smaller *Paramecium* population densities established on day 8, which were restricted due to competition with *Colpidium*. Therefore, competition could be said to further reduce the ability of *Paramecium* to persist under predation. Additionally, the fact that *Paramecium* went extinct faster in the predation treatment with *Colpidium* present could suggest that despite the smaller population density of *Paramecium,* Didinium selectively preferred *Paramecium* as prey over *Colpidium*. *Colpidium* was also negatively impacted by the competition with *Paramecium*. Despite the early extinction of *Paramecium*, the *Colpidium* population density remained lower than in the *Colpidium-Didinium* treatment throughout the course of the experiment. This suggests that despite collapsing to the same community the initial presence of *Paramecium* had a lasting effect upon the community. The lower density of *Colpidium* where *Paramecium* was initially present could have been directly due to the early competition. However, another explanation could be that the early presence of *Paramecium* caused the *Didinium* population to reach a much higher population density, and therefore increased the effect of predation upon *Colpidium* through apparent competition.

Temperature impacts the timings of extinctions and community stability

Following the metabolic theory of ecology, it stands to reason that increasing temperature causes an increase in metabolic rate, thus causing individuals to utilise resources at a faster rate at higher temperatures (Brown *et al.,* 2004, Clements *et al.,* 2014). Considering the average population densities of *Didinium* in the *Colpidium-Didinium* community, the results suggest that the *Didinium* populations remain fairly stable until the end of the experiment. However, considering individual replicates it appears that there is an effect of temperature upon the number of extinctions. By day 39, no extinctions of *Didinium* were recorded at 22°C, one extinction was recorded at 24°C and four extinctions were recorded at 26°C. This temperature response is likely to reflect the effect of a lower *Colpidium* population density at higher temperatures,

combined with a higher metabolic cost associated with higher temperatures causing *Didinium* to be a less sustainable population at higher temperatures. Additionally, considering the extinction of *Paramecium* and *Didinium* in the *Paramecium-Didinium* community on a replicate basis reveals an effect of temperature upon stability. For both *Paramecium* and *Didinium*, extinction occurred faster at higher temperatures. These results suggest that extinctions occurred faster with increasing temperature, thus temperature negatively affects stability in terms of persistence.

3.4.2 The bacterial community

These results highlight the responses of the bacterial community to manipulations in trophic structure and temperature. The very low density of *B. cereus* could have been the result of competition with *P. fluorescens* and *S. marcescens.* However, it is also a possibility that the environmental conditions were unfavourable for *B. cereus.* In the bacteria-only community *P. fluorescens* and *S. marcescens* reached and maintained very high densities. This suggests that the community did not exhaust the supply of energy. The population density of *S. marcescens* rapidly increased at 24°C and 26°C from day 12 to day 29. This response could be a result of an advantageous morph of *S. marcescens* enabling the species to utilise more resources or utilise resources more efficiently. Determining the mechanism responsible for this boost in density requires further investigation.

The addition of bacterivores, *Paramecium* or *Colpidium*, significantly reduced bacterial abundance (fig. 3.9, 3.10). Without the presence of *Colpidium*, *P. fluorescens* was the most abundant bacteria at 22°C and 24°C, and for the first 22 days at 26°C before *S. marcescens* became dominant. However, in the presence of *Colpidium, S. marcescens* and *P. fluorescens* had very similar abundances. The presence of *Colpidium* could have altered the bacterial assemblage through two mechanisms. Firstly, *Colpidium* could have reduced the competitive effect upon *S. marcescens*, boosting the population density of *S. marcescens* and thus enabling *S. marcescens* and *P. fluorescens* to persist at similar densities. Secondly *Colpidium* could have selectively consumed *P. fluorescens*, thus reducing *P. fluorescens* to the same density as *S. marcescens*. Predation by *Paramecium* or *Colpidium* appeared to have an equal effect upon the bacterial population, despite

different population densities and body sizes. This may be a surprising result, especially as *Colpidium* was 10-20 times more abundant than *Paramecium.* This suggests that the negative impact of one *Paramecium* individual upon bacterial abundance is equal to the negative impact of 10 – 20 *Colpidium.*

The presence of *Paramecium* and *Colpidium* together, did not form an additive effect of reduction in bacteria. This result supports the findings of Jiang and Krumins (2006) and provides evidence for the presence of multiple predator effects within a microbial community. Jiang and Krumins (2006) found that interference competition was likely between these two competitors and suggest that physical interference could force the competitors to alter their spatial niche and diet preferences. Studies have found that *Colpidium* is a specialist predator of *S. marcescens* and *Paramecium* is a generalist predator (Jiang and Morin, 2004, Jiang and Krumins, 2006). However, in this study the results provide evidence that *Colpidium* significantly reduced the abundance of both *S. marcescens* and *P. fluorescens.* It is possible that *Colpidium* and *Paramecium* can coexist by the efficient division of resources. Plus, both ciliates were negatively impacted by competitive interaction, so it may be that the overall reduction of bacterial abundance was roughly equal in the presence of one predator and in the presence of two predators, each at a smaller density.

The addition of a third trophic level relieved the pressure of predation upon the bacterial community, resulting in a higher bacterial abundance than in communities with two trophic levels. The magnitude of this effect was highly dependent upon community structure, species identity and temperature. For example, the strong temperature response of the population density of *Colpidium* was transferred through the network in the *Colpidium-Didinium* community. The negative temperature response of *Colpidium* meant that *Didinium* also exhibited a negative temperature response due to prey availability. By the end of the experiment, the mean population density of *P. fluorescens* was highest at 22°C, potentially due to the large *Didinium* population reducing the *Colpidium* However, the *S. marcescens* population did not echo this response. The *Paramecium-Didinium* community can be thought of as an unstable system, and effectively collapsed to a one trophic level system. However, bacterial abundance did not return to the same level of abundance as the bacteria-only community.

These results show the basal trophic level of bacteria responds to community structure and suggests that some bacteria species may be sensitive to species-specific temperature response in higher trophic levels.

3.5 Conclusion

In summary, this study has found evidence for the following conclusions:

1) Community response to temperature is strongly dependent upon specific-species temperature response and is mediated though the community via both, direct and indirect, interactions.
2) The stability of predator-prey communities is highly dependent on prey species identity.
3) Temperature affects the timing of extinction events, with higher temperatures causing faster extinctions.
4) The bacterial community exhibits dynamics which are highly dependent upon community structure and the population dynamics of higher trophic levels.

4 Discussion

In this study I have used experimental microcosms and molecular techniques to investigate the effects of temperature upon species interactions. In Chapter 2, I developed the application of the qPCR technique to bacteria in experimental microcosms. In Chapter 3, incorporating this technique, I investigated the effects of temperature and community structure upon three trophic levels. I will briefly review the research completed investigating species interactions and climate change and my key findings from Chapter 3. I will also review the practicality of the qPCR technique developed in Chapter 2 and potential applications for the technique in future.

4.1 Species interactions and climate change

Altermatt *et al.,* (2015) consider identifying, understanding and causally linking processes within an ecosystem as the greatest challenge facing ecologists today. The shift in focus to a mechanistic understanding of species environmental response and species interactions has led to the development of the MTE and foraging theory. The fundamental links between body size, temperature and biological processes (Brown *et al.,* 2004, Gillooly *et al.,* 2001, Petchey *et al.,* 2008) enable the testing and modelling of the mechanisms that may underpin species interactions and response. However, expanding this mechanistic approach to community-level responses presents a challenge (Montoya and Raffaelli, 2010). As ecologists we still need to resolve our understanding of the impact of separate components of climate change and their potential interactions, upon populations and communities.

The experiment discussed in Chapter 3 enabled the contrast of a highly unstable community (*Paramecium-Didinium)* and a relatively stable community (*Colpidium-Didinium)*. The difference in stability therefore appears to be due to prey-specific traits. These traits could include the prey's body size, swimming speed, and growth rate. Furthermore, determining the direct effects of temperature upon *Didinium* would allow the evaluation of the impact of prey species temperature-response. By creating cultures of *Didinium* and regularly feeding the population the direct effects of temperature and prey availability could be quantified. Investigating these traits directly could develop our understanding of predator-prey stability.

As previously discussed in Chapter 1, environmental factors rarely act in isolation. Understanding the effects of environmental factors individually could build a mechanistic understanding, which could be based on the MTE and foraging theory. Griffiths *et al.,* (2015) used an experimental protist microcosm system to factorially manipulate temperature, salinity and productivity to examine the interaction of these environmental factors. Adopting this experimental design presents an excellent opportunity to investigate the effects of multiple environmental conditions and could build upon the research undertaken so far to determine the effects of individual environmental parameters. This would allow the determination of whether effects are additive and how environmental factors, and the resulting responses interact.

4.2 Future applications of qPCR

As discussed in Chapter 2, molecular techniques, including PCR and qPCR, have been developed and applied to bacteria in food microbiology, wastewater and soil ecology. Considering molecular tools are used elsewhere, it seemed the toolbox for experimental microcosms could be updated. This research has developed a technique which can improve the accuracy of monitoring the bacterial community within experimental aquatic microcosms. Utilising qPCR in this approach to investigate species interactions in a community can aid our mechanistic understanding of these experimental systems. Chapter 3 suggests that in these experimental systems there are strong interactions between the three trophic levels. With the ability to collect quantitative bacterial data, a next step would be to integrate the approach of experimental microcosms and mathematical models. Utilising the data gained through qPCR in mathematical models could aid the modelling of interactions across three trophic levels. This could then aid the prediction of community responses to perturbations such as environmental change.

There are still areas of development for the qPCR method, observed in Chapter 2 and Chapter 3. It would be useful to test more bacterial species to select species that are less inclined to form chains and aggregates. The species of bacteria used here were chosen because they are commonly used in microcosm experiments (see table 1.1). Species were often selected to be morphologically distinct and readily culturable on agar plates. However, their selection pre-dates the molecular techniques developed here and it may be possible to find species that are equally amenable to manipulation in microcosms, but are easier to sample by qPCR. It could also be advantageous to explore the use of media that does not contain particulate matter. The chlorella media used here appears similar in composition to the Protist Pellet supplied by Carolina Biological Supply (which is used by several research groups; see Balciunas and Lawler, 1995, Jiang and Morin, 2004, Hammill *et al.,* 2015), but makes sampling bacterial communities harder than a fully soluble media. The down-side to such media is that it is the particulate nature of these media that facilitates the very long-term community dynamic that microcosm communities are so good at modelling. In future to collect more consistent data for the bacterial trophic level using qPCR, scaling down the scope of the ecological experiment would allow retesting of samples where the qPCR reaction failed to produce anything or

produced a non-specific product, classified as a fail or NA. It was not possible to revisit failed samples at the time of sampling and testing due to time constraints and the limited amount of qPCR reagents. Developing the ability to freeze samples for qPCR would enable a wider scope for an experiment by making more time available on each sampling day when qPCR could be completed at the end of the experiment. Developing cheaper self-made reagents, such as phusion polymerase (see Appendix A) would also reduce the cost of the method and enable resources to stretch further.

Initially it was intended that the qPCR methodology would enable the rigorous testing of the interaction between temperature and community properties, including connectance. However, there were some limitations for this application found during the development of the qPCR methodology in Chapter 2. The tendency of *B. cereus* to form chains and the interference with the qPCR due to this characteristic was unexpected. In Chapter 3, the *B. cereus* population fluctuated in and out of detectability. This meant that some sampling days recorded a false absence of *B. cereus*. Developing a method to determine the complete extinction of a bacterial species would enable the investigation of lower trophic level extinctions and network properties such as connectance and connectivity distribution.

It should be noted that the organisms used in Chapter 3 do not have complex life stages or a population structure. Without these characteristics, the simplicity enables the focus to be placed upon the effects of temperature and community structure. However, in nature structured population with complex life-history traits are common. On the other hand, the bacteria populations used in the 'simplistic' system in Chapter 3, may not be as simplistic as imagined. Several morphotypes were observed for each bacterial species (fig. 2.13, 2.15) and the formation of biofilms appeared to be influenced by the presence of particulate matter and also the presence of a predator. The strain (SBW25) of *P. fluorescens* in this study has been previously investigated as a model organism to research adaptive radiation (Rainey and Travisano, 1998). It is acknowledged that bacteria, and microbes, can exhibit rapid evolutionary change due to their rapid generation time and large population size (Spiers and Rainey, 2005). Rainey and Travisano (1998) note that diversification can be dependent on spatial heterogeneity to create an ecological opportunity. Additionally, Meyer and Kassen (2007) reported that the presence of a predator, *Tetrahymena thermophila*, affected the diversification of *P. fluorescens.* Friman *et al.,* (2008) found evidence that *S. marcescens* decreases motility

and forms biofilms in a defensive evolution in response to predation by *T. thermophile*. The microscopic imaging in Chapter 2 supports this assertion, as it was found that different morphotypes of bacteria formed in media with particulate matter than in soluble media. Considering the potential for diversification and the morphotype characteristics associated, it is perhaps surprising that the bacterial trophic level has been largely unmonitored and reported as an aggregated simplistic community – the bacterial black box.

4.3 Collaborative research and development

There are different approaches and methodologies used to investigate ecological principles and processes, including mathematical models (Vandermeer, 1969, Beckerman *et al.,* 2006, Englund *et al.,* 2011), laboratory experiments (see table 1.1), field experiments (reviewed in Gurevitch *et al.,* 1992, 2000) and natural observational studies. These approaches can all offer clarity to our understanding of ecological systems (Benton *et al.,* 2007, Dunne *et al.,* 2002). However, each methodology has a distinct set of advantages and limitations, particularly when judged upon generality, spatial scale, scope, regulation and replication (fig. 4.1, Woodward *et al.,* 2010b). Yet these are often overlooked, misunderstood or controversial (Diamond and Case, 1986). The singular development of these methods has led to an isolated stance of ecologists with some principled claims of superiority. Arguments supporting a particular method often focus on its advantages, disregard the method's limitations and emphasise other methods restrictions whilst ignoring any benefits (Diamond and Case, 1986). Benton *et al.,* (2007) states that the deep division between methodologies inhibits development and these seemingly antagonistic approaches could gain much by working synergistically. To accelerate progress, it is critical to develop an integrated research strategy which would produce more robust conclusions and ultimately a greater comprehension of ecological principles.

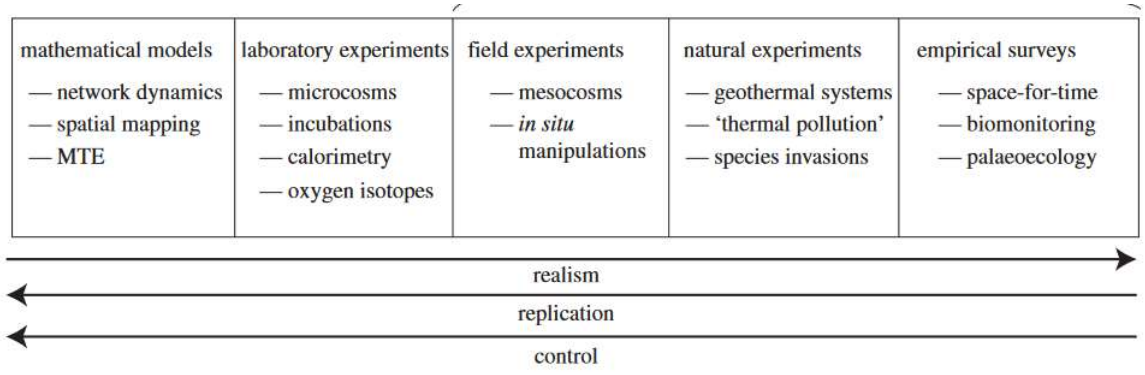


Figure 4.1: Adapted from Woodward *et al.,* (2010b). Schematic of methodological approaches, shown along a gradient of realism, replication and control.

4.4 Concluding statement

Disentangling the response of a community to temperature change remains a major challenge in ecology, that will require collaborative research for empirically testing theory, developing predictive mathematical models and the ultimate application to natural communities. Developing a more advanced toolbox for each approach is advantageous to rapidly and accurately improve our mechanistic understanding of community response. Developing the qPCR approach in Chapter 2 was an exciting process, albeit with some challenges such as the aggregation of bacteria in chains and to particulate matter and the complex nature of morphotypes of bacteria. However, in itself, these challenges and limitations of the technique have led to interesting research questions. Applying the qPCR method in an ecological experiment in Chapter 3 tested the method's practicality and suggests a promising future for qPCR in this context. From the evidence found in Chapter 3, the effects of temperature upon a community are highly dependent on specific species responses, indirect interactions and the community structure. Not accounting for the indirect pathways that mediate temperature response and the effects of community structure at all trophic levels could lead to misinterpreting community response and false predictions for the future. While our climate and environment continue to change, with the development of a qPCR approach, I hope the bacterial black box in microcosms can be explored, and collaborative research will accelerate the progression of our understanding.

Appendix A: Polymerase

The PCR reaction requires a heat-stable enzyme to act as the DNA polymerase. The most commonly used is 'Taq' polymerase. Taq polymerase is an enzyme originally isolated from *Thermus aquaticus.* Phusion 'pfu' polymerase is an alternative thermostable enzyme isolated from the species *Pyrococcus furiosus* in a vector. The gene was cloned and recombinantly expressed in *Escherichia coli* using a T7/Lac promoter gene. Isopropyl-B-D-thiogalactopyranoside (IPTG) is a synthetic analogue of lactose and can be used to induce the expression of the enzyme (Hu et al., 2014). Phusion has a higher thermo-tolerance than taq and pfu possesses proofreading activity, therefore pfu is superior to taq.

To optimise the reagents for the qPCR process and investigate methods of cutting costs, phusion polymerase was produced and tested against taq polymerase. This was achieved using an *Escherichia coli* strain (Rosetta 2 DE3) that has been manipulated to express the phusion enzyme when the production pathway is induced. Phusion is a modified Pfu polymerase with a DNA binding domain added to increase productivity. The pathway is induced when the strain is stimulated to use the lac operon system. As mentioned IPTG (Isopropyl β-D-thiogalactopyranoside) is a supplement for lactose which the bacteria cannot digest but will still induce the pathway. This is used because if the IPTG is not being digested it will not become a limiting factor in the production of the phusion enzyme. Kanamycin was added to LB media to act as a selective agent to ensure the isolation of *E. coli* which has been transformed to be kanamycin resistant and possess the pathway which produces the phusion enzyme.

The procedure for the production of the phusion enzyme involved growing a 1OD culture of the *E. coli* containing the vector, inducing the production of phusion by adding IPTG and then isolating the phusion enzyme. The procedure was monitored and assessed via the production of an SDS Page gel. The details of this procedure are outlined below:

1. Initially the *E. coli* was added to 5ml of LB media with 5µl of kanamycin 100mg/ml and grown overnight at 37°c.
2. The following morning a sample was taken from this dense 5ml culture and retained for future use.
3. The remainder of the culture was added to 500ml of LB with 25mg of kanamycin and grown at 37°c for three hours in a shaking incubator.
4. From this culture 125µl was sampled and spun for 1 minute. The supernatant was then removed and 20µl of water and 20µl of 4x SDS loading buffer added. The sample was then heated for 4 minutes at 95°c. This sample was labelled 'uninduced' and stored at 4°c, ready to be used for the SDS Page gel at the end of the phusion production process.
5. 54mg of IPTG was added to the dense culture of *E. coli* and grown for 3 hours at 37°c in a shaking incubator. The addition of IPTG induces the production of the phusion enzyme.
6. After the 3 hours, another 125µl from this culture was sampled and spun for 1 minute. The supernatant was removed and the sample was resuspended in 20µl of water and 20µl of 4x SDS loading buffer. This sample was then heated for 4 minutes at 95°c. This sample was labelled 'induced' and also stored at 4°c, ready for the SDS page gel.
7. The dense culture was split into 50ml centrifuge tubes and spun for 10 minutes at 4900rpm at 21°c. This caused the formation of pellet containing the cells. The supernatant was carefully removed without disturbing this pellet and the samples were then frozen for storage.
8. The next day one of these pellets was thawed and resuspended in 4ml of LEW buffer. The mixture was then heated in a water bath at 70°c for 15 minutes.
9. After this heating, the mixture was spun at 10000rpm for 20minutes, moving the insoluble back into a pellet and producing a supernatant rich in phusion DNA protein.
10. A 20µl sample of the supernatant was taken and 20µl of 4xSDS buffer with 20µl of water added. This sample was labelled 'post-boil'. This sample was then heated at 95°c for 4 minutes.
11. The rest of the supernatant was then removed and put through a Ni-IDA column.

Ni-IDA column:

- 2ml of LEW buffer, acts as waste.
- All the supernatant and kept as 2ml aliquots.
- 2x 2ml LEW, wash 1 and wash 2, W1 and W2.
- 5x 1ml Elution buffer, E1 E2 E3 E4 E5.

The initial 2ml of LEW buffer is waste but acts to wet the column. The supernatant flows through the column, and the tagged phusion proteins become attached to the poly-histidine tagged proteins and remain within the column. The LEW buffer used as a wash, then removes all excess waste that has not bound without displacing the desired protein from the column. The elution buffer then washes the column replacing the protein in the poly-histidine bonds and releasing the bound protein from the column.

To check the purity of the samples, all samples were run on an SDS-PAGE gel (fig. A.1). A 20µl sample from each was taken and 20µl of 4x SDS buffer added. The samples were then heated for 4 minutes at 95°c, the same as the samples labelled uninduced, induced and post-boil.

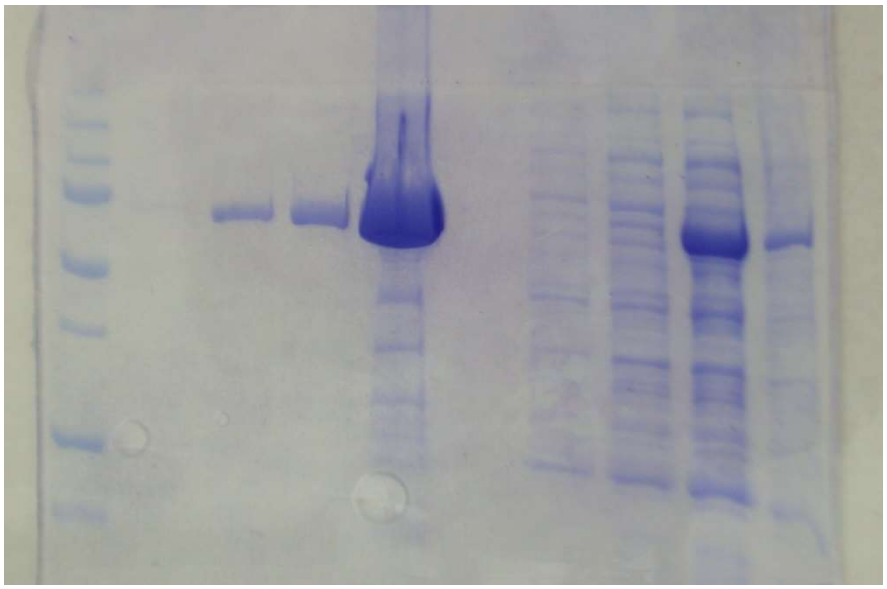


Figure A.1: SDS Page Gel showing a ladder (lane M), uninduced, induced, post-boil, wash buffer (W1, and W2) elution buffer with phusion protein, (E1, E2, E3, E4 and E5).

After filtration the phusion protein was held in an aqueous solution in the elution buffer. To isolate the protein a ‘salting out’ process was undertaken. By adding ammonium

sulfate, the protein's solubility is reduced, causing the protein to coagulate and be selectively precipitated. These samples can then be centrifuged to form a pellet, rich in the protein. Once the supernatant is removed the pellet can be resuspended in LEW buffer. To ensure the protein remains in the solution at a high concentration, the sample is placed within a dialysis membrane overnight**.**

Using *E. coli* primers and sample as a control reaction with Taq allows the comparison of phusion and taq and tests the performance of phusion at different concentrations. In figure A.2, it can be seen that the control reaction (lane 1) formed a band of ~600bp. The reactions with phusion at full concentration, and 1/5 dilutions did not produce a band (lanes 2 and 3), whilst phusion at a dilution of 1/25 and 1/125 produced clear bright bands (lanes 4 and 5). This showed successful production of active protein.

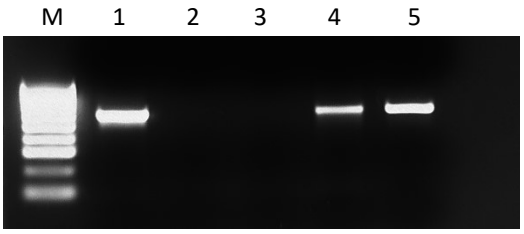


Figure A.2. Agarose gel electrophoresis comparing Taq and different concentrations of phusion, using *E. coli* and control primers (Forward primer: TGCGTCACGTAAAACTGCATC and Reverse primer: ACCGTCTTTCACTTCAACGGT). The lane marked 'M' is the 100bp molecular weight marker, lane 1 shows taq with *E. coli* control primers, lanes 2-5 shows phusion with *E. coli* control primers at full concentration, and dilutions of 1/5, 1/25 and 1/125.

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DETERMINING THE EFFECT OF TEMPERATURE ON SPECIES INTERACTIONS IN MICROCOSMS: A QPCR APPROACH

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